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☐	L12	L11 same mole	6
☐	L13	edta near3 calculate near3 molarity	0
☐	L14	1 near2 mm near2 edta	16498
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EDTA

From Wikipedia, the free encyclopedia.

EDTA is the chemical compound **ethylenediaminetetraacetic acid**. EDTA or its disodium salt is a chelating agent, forming coordination compounds with most divalent (or trivalent) metal ions, such as calcium (Ca^{2+}) and magnesium (Mg^{2+}) or copper (Cu^{2+}).

Uses

- Scavenging metal ions
- Complexometric titrations
- Buffer solutions
- Determination of water hardness
- Use as a water softener.
- Used in medicine as a treatment for acute hypercalcemia and lead poisoning.
- Used in medical and laboratory equipment as an anticoagulant.
- Added to some processed foods and especially cosmetics as a preservative.
- A somewhat controversial therapy, called chelation therapy has evolved around metal scavengers such as EDTA.
- Used in dentistry as a root canal irrigant to remove compounds of organic and inorganic debris (smearlayer)
- Used in photography as a component of bleach-fix used to disolve elemental silver produced during development.
- Used as a soil conditioner to allow cacifuge plants to grow in base rich soils

Use as an anticoagulant

EDTA works as an anticoagulant by chelating all the calcium contained in blood. Calcium is needed for coagulation to occur; without calcium blood will not clot. The calcium levels below which clotting ceases are low enough to be lethal, so EDTA is only used as an anticoagulant outside the body; for instance in tubes of blood, and medical machinery.

EDTA is contained in purple, lavender and pink Vacutainer (tubes that blood is taken in), and can be in the form of a powder, or a small amount of liquid, already in the tube.

The sodium or potassium salts of EDTA (K_2EDTA, K_3EDTA, Na_2EDTA) are used in Vacutainer tubes. This means levels of these ions are increased, and detectable levels of calcium and magnesium are decreased. For this reason many clinical chemistry tests are not done using plasma from EDTA tubes.

External links

- pH-Spectrum of EDTA complexes (*http://www.theoprax-research.com/pool.html*)

Categories: Antidotes

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L17: Entry 136 of 166 File: USPT May 21, 1996

DOCUMENT-IDENTIFIER: US 5519115 A
** See image for Certificate of Correction **
TITLE: Reverse antimicrobial peptides

Brief Summary Text (7):
Others have published information relating to the existence of antimicrobial peptides in plants or, in fact, the use of antimicrobial peptides to protect plants from plant pathogens. See, EPO 0,299,828; P. Casteels et al., "Apidaecins: Antibacterial Peptides From Honeybees," The EMBO J. 8, (1989), 2387-2391; F. Ebrahim-Nesbat et al., "Cultivar-Related Differences in the Distribution of Cell-Wall-Bound Thionins in Compatible and Incompatible Interactions Between Barley and Powdery Mildew," Planta 179, (1989), 203-210. Most of this work centered upon the identification and use, either in plants or animals, of basically full-length natural antimicrobial peptides.

Detailed Description Text (6):
While the term may have broader application to one or more entire families of antimicrobial peptides, antimicrobial peptides active against at least one pathogen and the term AMPP encompass: Magainin 1; Magainin 2; reverse magainins; P1 and reverse P1; PGL.sup.c and reverse PGL.sup.c ; Cecropins including Cecropins A, B, and D and their reverse peptides, see H. G. Boman et al., "On the Primary Structures of Lysozymers, Cecropins, and Attacins from Hyalophora cecropia," Developmental and Comparative Immunology, 9, (1985), 551-558; Sarcotoxins and their reverse peptides; Bombinins and their reverse peptides, see A. Csordas and H. Michl, "Isolierung und Strukturaufklarung eines Hamolytisch wirkenden Polypeptides aus dem Abwehrsekret europaischer Unken," Monat fur Chemie, 101, (1970), 182-189; XPF and its reverse peptide; Thionins and their reverse peptides; Defensins and their reverse peptides, see H. Vogel et al., "The Structure of Melittin in Membranes," Biophys. J., 50, (1986), 573-582; Melittins and their reverse peptides, see H. Vogel et al., "The Structure of Melittin in Membranes," Biophys. J., 50, (1986), 573-582; and other equivalent peptides and functional derivatives thereof.

Detailed Description Text (25):
In accordance with the present invention, RAMPPs can be produced which have the identical but reversed sequence of peptides including reverse Magainin 1 (SEQ ID NO. 9), reverse Magainin 2 (SEQ ID NO. 10), reverse P1 (SEQ ID NO. 13), reverse Cecropin A (SEQ ID NO. 14), as well as reverse PGL.sup.c (SEQ ID NO. 7), and reverse forms of other Cecropins, Sarcotoxins, Bombinins, XPF, Thionins, Defensins, Melittins, and like antimicrobial peptides.

Detailed Description Text (32):
Other peptide monomers useful in accordance with the present invention are Magainin 1 having a structure of (SEQ ID NO. 1), Magainin 2 having a structure of (SEQ ID NO. 2), P1 having a structure of (SEQ ID NO. 6), PGL.sup.c having a structure of (SEQ ID NO. 7), Cecropins such as Cecropin A having a structure of (SEQ ID NO. 8), Sarcotoxins, Bombinins, XPF, Thionins, Defensins, Melittins, and like antimicrobial peptides.

Detailed Description Text (160):
Any cleaved and deprotected oligonucleotides prepared by methodology drawn from the preferred choices above can be purified by one or more of several methods known in

the art. These purification techniques include but are not limited to polyacrylamide polyacrylamide gel electrophoresis, agarose gel electrophoresis, size exclusion chromatography, affinity chromatography, high performance liquid chromatography and hydrophobic interaction chromatography. The preferred method is selected from the group of purification techniques which consists of polyacrylamide gel electrophoresis, high performance liquid chromatography and hydrophobic interaction chromatography. One such preferred method is polyacrylamide gel electrophoretic purification of oligonucleotides, lacking a dimethoxytrityl moiety on the 5' terminus, on a vertical 12% polyacrylamide slab gel, 20.times.40.times.0.08 cm, in 7M urea, 90 mM Tris-HCl, pH 8.3, 90 mM borate, 1-2 mM disodium ethylenediaminetetraacetic acid (EDTA). A portion of each oligonucleotide to be purified (0.3-3.0 A.sub.260 units) is evaporated to dryness under vacuum, resuspended in formamide:1 mM disodium EDTA (greater than 9:1) containing at least 0.01% bromophenol blue and at least 0.01% xylene cyanol, heated 2-3 minutes in a boiling water bath, quickly placed in an ice slurry and then loaded in an individual well (at least 6 mm in width). The sample(s) is electrophoresed at 80-90 W towards the anode until the bromophenol blue has migrated at least two-thirds the length of the polyacrylamide gel. The full-length oligonucleotides are then visualized by placing the polyacrylamide gel on a piece of flexible, clear plastic wrap such as Saran Wrap, placing it on top of a thin layer chromatography plate (e.g., Silica Gel F-254; Fisher Scientific Company, Pittsburgh, Pa.) containing a fluorescent indicator compound and examining the polyacrylamide gel under short wavelength ultraviolet light illumination. The full-length band material is then excised in polyacrylamide and can be purified out of the gel by various methods such as electroelution or simple diffusion in buffer. The preferred method of extraction is diffusion into 0.5 mL of 0.3M sodium acetate, pH 7.5, overnight with shaking and successive extractions of the aqueous phase with phenol:chloroform (1:1, v:v) and ethanol precipitation. The precipitated oligonucleotide can then be resuspended in an appropriate volume (usually in the range of 10-1000 microliters) of a suitable buffer such as 10 mM Tris-HCl, pH 7.5, 1 mM disodium EDTA or in sterile distilled water and stored at -20.degree. C. See unit 2.12, "Purification of oligonucleotides using denaturing polyacrylamide gel electrophoresis," in Current Protocols in Molecular Biology, F. M. Ausubel et al., Eds., (1989).

Detailed Description Text (260):
In order to obtain an enriched chloroplast fraction, about 12 g of deribbed spinach leaves were thoroughly washed and surface dried. The leaves were then cut into small pieces, each about 1/2 inch square, and were placed in a small blender jar containing 50 mL of chilled homogenization medium (0.33M sorbitol, 50 mM Hepes-NaOH, pH 6.8, 2mM Na.sub.2 EDTA, 1 mM MnCl.sub.2, 1 mM MgCl.sub.2). The tissue was blended twice for three second intervals on high speed in a blender. The resulting homogenate was filtered through four layers of cheesecloth and two layers of miracloth (Behring Diagnostics, La Jolla, Calif.) into two chilled 30 mL glass centrifuge tubes. The filtered solution was centrifuged for 1.0 minute at 750 g (2,200 RPM) in a JS 13.1 swinging bucket rotor in a Beckman J2-21M centrifuge (Beckman Instruments, Inc., Somerset, N.J.). The supernatant was then decanted and the pellet was gently resuspended by swirling at 0.degree. C. About 15 mL of homogenization medium was added to each tube of chloroplasts before the chloroplasts were layered onto a 40% Percoll gradient (6 mL Percoll, 9 mL homogenization medium, and 0.03 g bovine serum albumin) in a 30 mL glass centrifuge tube. These tubes were centrifuged for 4.0 minutes at 2500 g (4000 RPM) in a JS 13.1 swinging bucket rotor in a Beckman J2-21M centrifuge. The supernatant was removed and the resulting pellet was resuspended in a small amount of homogenization medium (about 500 microliters).

CLAIMS:

1. A compound comprising a peptide which is a reverse antimicrobial peptide active against at least one microbial pathogen, said peptide having at least about an equal degree of resistance to proteolytic degradation when compared to a peptide of

the identical sequence in the opposite order and wherein said reverse antimicrobial peptide is selected from the group consisting of reverse Magainins, reverse PGL.sup.c, reverse P1's, reverse Cecropins, reverse Sarcotoxins, reverse Bombinins, reverse XPFs, reverse Thionins, reverse Defensins, reverse Melittins, and reverse PGL.sup.a.

19. The composition of claim 1 wherein said reverse antimicrobial peptide is a reverse Thionin.

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USE OF THE THIONINE–EDTA SYSTEM IN PHOTOGALVANIC CELLS FOR SOLAR ENERGY CONVERSION

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Summary

A photogalvanic cell, using thionine as a photosensitizer and EDTA as a reductant, was used for solar energy conversion. The photocurrent and photopotential generated by this cell were 80 μA and 900 mV respectively. The effect of various parameters on the electrical output of the cell was studied. Current–voltage characteristics of the cell were also investigated and a tentative mechanism for the generation of photocurrent in the photogalvanic cell was proposed.

1. Introduction

Rideal and Williams [1] discovered the photogalvanic effect in 1925, but it was systematically investigated by Rabinowitch [2]. Later, this type of work was carried out by various researchers [3 - 12] all over the world. Various problems encountered in the development of this field of research have been discussed by Hoffman and Lichtin [13]. The theoretical conversion efficiency of a photogalvanic cell is about 18% [14], but the observed conversion efficiencies are quite low. This may be due to back electron transfer, low stability of dyes, aggregation of dye molecules around the electrode, etc.

A critical survey of the literature reveals that different photosensitizers, such as proflavin [15], thionine [16], riboflavin [11], methylene blue [17, 18], brilliant cresyl blue [19], etc., have been used in photogalvanic cells. However, the use of thionine in photogalvanic cells for solar energy conversion has not been investigated. Therefore in this work we report the results of a study on the use of the thionine–EDTA system in photogalvanic cells for solar energy conversion.

1010-6030/89/$3.50

2. Experimental details

Thionine (Th) (Rideal), sodium hydroxide (SM) and EDTA disodium salt ('EM'GR) were used in this work. Doubly-distilled water was used to prepare all the solutions. A mixture of solutions of thionine, sodium hydroxide and EDTA was placed in an H-shaped glass cell. A platinum electrode (1.0 cm × 1.0 cm) was dipped in one limb of the cell and a saturated calomel electrode (SCE) in the other. The limb containing the platinum electrode was exposed to a 200 W tungsten lamp (Sylvania) and the limb containing the SCE was kept in the dark. A water filter was used for cutting out thermal radiation.

The photochemical bleaching of thionine was studied potentiometrically. The photopotential and photocurrent generated by the system Th/EDTA/OH^-/$h\nu$ were measured with a digital pH meter (Systronic, model 335) and multimeter (Systronic, model 435) using Pt–SCE and Pt–Pt electrode systems respectively. The I–V characteristics of the cell were observed using an external load (log 500 K) in the circuit.

3. Results and discussion

3.1. Effect on pH on electrical output of the cell

The effect of variation in pH on photopotential and photocurrent was studied and the results are given in Table 1.

TABLE 1

Variation in pH ([EDTA] = 8.40×10^{-4} M; [thionine] = 1.47×10^{-4} M; intensity = 10.4 mW cm^{-2}; temperature = 303 K)

pH	*Photopotential* (mV)	*Photocurrent* (μA)
10.9	713.0	54.0
11.1	810.0	60.0
11.3	900.0	80.0
11.5	842.0	71.8
11.7	798.0	62.0

The electrical output of the cell increases with an increase in pH, reaching a maximum at pH 11.3. Further increase in pH results in a decrease in the electrical output. The pH at the optimum condition is related to the pK_a value of the reductant; the desired pH is higher than the pK_a value (pH > pK_a). This may be explained by the fact that the reductant is available in its anionic form, which is its better donor form.

3.2. Effect of reductant concentration

The dependence of the electrical output of the cell on the concentration of reductant was observed and the results are summarized in Table 2.

TABLE 2

Variation in EDTA concentration ([thionine] = 1.47×10^{-4} M; pH 11.3; temperature = 303 K; intensity = 10.4 mW cm^{-2})

[EDTA] ($\times 10^{-4}$ M)	*Photopotential* (mV)	*Photocurrent* (μA)
6.90	597.0	34.0
7.40	645.0	42.0
8.00	886.0	72.0
8.40	900.0	80.0
8.80	873.0	64.0
9.30	709.0	46.0

A low output is obtained for the lower concentrations of EDTA because a smaller number of reductant molecules are available for electron donation to dye molecules. The higher concentrations of reductant also result in a fall in electrical output because the large number of reductant molecules may hinder the dye molecules from reaching the electrode within the desired time limit.

3.3. Effect of variation in dye concentration

The electrical output of the cell is affected by the variation in dye concentration. The results are reported in Table 3.

TABLE 3

Variation in concentration of thionine ([EDTA] = 8.40×10^{-4} M; pH 11.3; temperature = 303 K; intensity = 10.4 mW cm^{-2})

[Thionine] ($\times 10^{-4}$ M)	*Photopotential* (mV)	*Photocurrent* (μA)
0.85	685.0	52.0
1.00	742.0	63.0
1.30	804.0	72.0
1.47	900.0	80.0
1.75	816.0	74.0
2.00	765.0	68.0
2.24	699.0	61.0

It is clear from the table that electrical output of the cell decreases with a decrease in dye concentration because a smaller number of dye molecules are available for excitation and subsequent donation of the electrons to the platinum electrode. However, the larger concentrations of dye also result

in a fall in power output as the intensity of the light reaching the dye molecules near the electrode decreases after absorption of the major portion of the light by dye molecules present in the path.

3.4. Effect of diffusing path length

H cells of different dimension were used to study the effect of variation in diffusing path length on the current parameters of the cell (I_{max}, I_{eq} and initial rate of generation of current). The results are given in Table 4.

TABLE 4

Variation in diffusing path length [thionine] = 1.47×10^{-4} M; [EDTA] = 8.40×10^{-4} M; intensity = 10.4 mW cm^{-2}; pH 11.3; temperature = 303 K)

Diffusing path length (mm)	*Maximum photocurrent* I_{max} (μA)	*Equilibrium photocurrent* I_{eq} (μA)	*Rate of initial generation of current* (μA min^{-1})
30.0	85.0	81.0	6.8
35.0	87.0	80.0	7.2
40.0	90.0	80.0	7.5
45.0	92.0	79.0	7.8
50.0	95.0	80.0	7.9

There is a sharp increase in photocurrent in the first few minutes of illumination, followed by a gradual decrease to a stable value I_{eq}. This type of photocurrent behaviour indicates an initial rapid reaction followed by a slow rate-determining step at a later stage.

On the basis of the effect of diffusing path length on the current parameters of the cell [7], it may be concluded that the leuco and semi-reduced forms of the dye and the dye itself are the main electrode-active species at the illuminated and dark electrodes respectively. However, the reductant and its oxidized products behave as the electron carriers in the cell diffusing through the path.

3.5. Effect of electrode area and temperature

The value of I_{max} increases with an increase in electrode area, but its effect on I_{eq} is negligibly small. The photopotential decreases with an increase in temperature, whereas the photocurrent increases with an increase in temperature. The increasing photocurrent with increasing temperature may be due to the decrease in the internal resistance of the cell at higher temperatures. The increase in photocurrent results in a corresponding decrease in photopotential. The rapid decrease in potential can be compensated by the corresponding increase in photocurrent and the power of the cell is enhanced to the same extent.

3.6. Current–voltage (I–V) characteristics, conversion efficiency and performance of the cell

The open circuit voltage V_{oc} and short circuit current I_{sc} were measured using a digital pH meter (keeping the circuit open) and multimeter (keeping the circuit closed). The current and potential values in between these two extremes (V_{oc} and I_{sc}) were recorded with the help of a carbon potentiometer (log 500 K) connected in the circuit of the multimeter through which an external load was applied.

It was observed that the $I–V$ curve of the cell deviates from its ideal regular rectangular shape. A point in the $I–V$ curve called the power point is determined where the product of the current and potential is at a maximum. The values of current and potential at the power point are represented as I_{pp} and V_{pp} respectively. The fill factor and conversion efficiency of the cell are 0.36 and 0.2802% respectively.

The performance of the cell was studied by applying an external load, which is necessary to obtain the current and potential at the power point, after removing the source of light. It was observed that the cell can be used in the dark at its power point for 49 min. A photovoltaic cell cannot be used in the dark even for 1 s; therefore the photogalvanic system (Th–EDTA) has an additional advantage in that it can be used in the dark.

3.7. Mechanism

On the basis of the results obtained, the mechanism of photocurrent generation in the photogalvanic cell can be represented as follows.

Illuminated chamber

$$Th \xrightarrow{h\nu} Th^*$$

$$Th^* + EDTA \longrightarrow Th^- + EDTA^+ (OxR)$$

At Pt electrode

$$Th^- \longrightarrow Th + e^-$$

Dark chamber

At electrode

$$Th + e^- \longrightarrow Th^-$$

$$Th^- + OxR \longrightarrow Th + R$$

Th, Th^-, R and OxR are thionine, its leuco form, reductant EDTA and its oxidized form respectively.

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Novel defensin subfamily from spinach (*Spinacia oleracea*)

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Abstract **Antimicrobial peptides (So-D1-7) were isolated from a crude cell wall preparation from spinach leaves (*Spinacia oleracea* cv. Matador) and, judged from their amino acid sequences, six of them (So-D2-7) represented a novel structural subfamily of plant defensins (group IV). Group-IV defensins were also functionally distinct from those of groups I–III. They were active at concentrations < 20 μM against Gram-positive (*Clavibacter michiganensis*) and Gram-negative (*Ralstonia solanacearum*) bacterial pathogens, as well as against fungi, such as *Fusarium culmorum*, *F. solani*, *Bipolaris maydis*, and *Colletotrichum lagenarium*. Fungal inhibition occurred without hyphal branching. Group-IV defensins were preferentially distributed in the epidermal cell layer of leaves and in the subepidermal region of stems.**

Key words: Antimicrobial peptide; Innate immunity; Plant defense; Plant defensin; Plant pathogen; Spinach

1. Introduction

Defensins are among the best characterized cysteine-rich antimicrobial peptides in plants (see [1,2] for a review). All known members of this family have 4 disulfide bridges and are folded in a globular structure that includes three β-strands and an α-helix [3,4]. This structure resembles that of antimicrobial defensins from insects [5,6]. Inhibition of fungal growth by plant defensins seems to occur by permeabilization of the plasma membrane through binding to a putative receptor [7,8].

Genes encoding plant defensins are developmentally regulated, with a predominant expression in outer cell layers [9–11], and can be induced above basal levels in response to pathogen infection and other stresses [10–14]. Additionally, certain defensin genes are down-regulated by some pathogens [10]. Gene expression patterns of defensins are thus consistent with a hypothetical role in plant defense [1,2]. The observation of enhanced tolerance (reduced lesion area) to the fungus *Alternaria longipes* in transgenic tobacco overexpressing a radish plant defensin (Rs-AFP2) further supports this hypothesis [11].

Known defensins have been classified into three groups or subfamilies [1,2,8,13,15], based on structural and functional considerations: group I includes defensins that inhibit growth of *Fusarium culmorum* and cause increased hyphal branching; group II includes those that inhibit the fungus but do not cause hyphal branching; and group III, those that are inactive against the tested fungi. Apart from highly conserved amino acid residues that are common to all three subfamilies, there are residues that are conserved only in one or two of them, which allows to discern a closer relationship between groups I and II than between any of these and group III.

We report here six new defensins isolated from crude cell wall preparations from spinach leaves which represent a novel defensin subfamily (group IV), both in structural terms and in its pathogen specificity. Defensins of this new type coexist with those of group III in the same tissue.

2. Materials and methods

2.1. Purification and analysis of proteins

Spinach, *Spinacia oleracea* cv. Matador, was used in this study. Frozen leaves (20 g) were ground to powder in liquid nitrogen, using a mortar and pestle, and extracted once with 80 ml buffer (0.1 M Tris-HCl, 10 mM EDTA, pH 7.5) and twice with 80 ml of H_2O. The resulting pellet was then extracted with 50 ml 1.5 M LiCl at 4°C for 1 h, and the extract dialyzed against 5 l H_2O, using a Spectra/Por 6 (MWCO: 3000) membrane, and freeze-dried [16]. The extract was fractionated by reverse-phase HPLC (RP-HPLC) as previously described [16]. The proteins were subjected to SDS-PAGE in preformed gradient gels (4–20%; Bio-Rad) according to the manufacturer's instructions. MALDI mass spectrometry of proteins was done in a Voyayer Biospectrometry Workstation (PerSeptive Biosystems) using α-cyano-4-hydroxycinnamic acid (Aldrich) as matrix. Amino acid sequencing of intact proteins or of chymotryptic peptides was done by automated Edman degradation. Detection of proteins by Western-blot and by the tissue-print technique was done as previously described using an 1:500 dilution of the So-D2-7 antiserum [17].

2.2. Pathogen inhibition tests

Inhibition tests were carried out as previously described [16] and hyphal branching of *Fusarium culmorum* was investigated as indicated by Broekaert et al. [18]. The following microbial strains were used: bacterial pathogens *Clavibacter michiganensis* subsp. *sepedonicus* strain C5, *Ralstonia solanacearum* strain P2; and fungal pathogens *Fusarium solani* strain 1, and *Trichoderma viridae* from the ETSIA collection (Madrid, Spain), and *Septoria nodorum*, *Bipolaris maydis*, *Colletotrichum lagenarium* and *Fusarium culmorum* from the collection of Novartis (NC, USA).

3. Results

3.1. Purification of spinach defensins

A crude cell wall preparation from spinach leaves was obtained as previously described [16,19,20] and bound proteins were extracted with 1.5 M LiCl. The extract was fractionated by RP-HPLC as shown in Fig. 1A and the fractions screened for antibacterial activity at 100 μg/ml. Homogeneity of active fractions was tested by SDS-PAGE and by RP-HPLC, using a less steep gradient. All active fractions were homogeneous according to these two criteria, except for the fraction designated (2–5) in Fig. 1A that yielded 4 homogeneous components upon rechromatography (Fig. 1B,C). The purified pro-

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teins, So-D1-7, also appeared homogeneous when subjected to MALDI mass spectrometry analysis and to N-terminal amino acid sequencing. A gene-bank search indicated that all of these proteins were homologous to previously reported plant defensins and a comparison of all known sequences showed that six of the new proteins, So-D2-7, appeared to represent a new defensin subfamily, whereas protein So-D1 belonged to the previously proposed group III (Fig. 2A,B). The complete amino acid sequence of So-D2 was determined after chymotryptic digestion and the MW calculated from this sequence (5804 Da) was within 1 Da of that directly determined by MALDI mass spectrometry. The new group is structurally closer to group III than to groups I and II, but shows divergence from group III at the N-terminal half, including a 5-residue extension. Some common amino acid residues are shared by defensins of groups I and II with drosomycin, a defensin from the insect *Drosophila melanogaster* [6], and by those of groups III and IV with tenecin, a defensin from the insect *Tenebrio molitor* [22].

3.2. Distribution of group-IV defensins in the plant

Rabbit antiserum raised against protein So-D2 recognized proteins So-D2-7 and did not significantly bind protein So-D1 (Fig. 3). Using this antiserum, group-IV defensins were detected in spinach leaves and stems (not in roots) at concentrations that were in the range of 1–3 μmol/kg of fresh weight in the homogenized proteins (Fig. 3). As shown by tissue-print analysis, the distribution of the proteins in these tissues was peripheral, as they were at higher concentrations in the epidermal cell layer of leaves and occupied a wide subepidermal band in stems (Fig. 4). The actual concentrations in the deposition sites are probably up to 10-fold higher, well above the concentrations required for inhibition in vitro. As judged from the tissue prints of young and mature leaves (Fig. 4B,C), the protective defensin shield seems to be present throughout the life of this organ. This type of distribution is common to other defensins and antimicrobial peptides from plants [1].

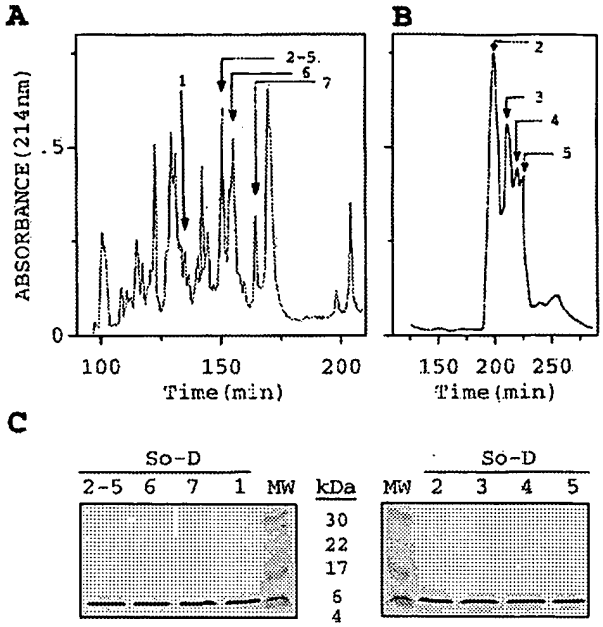

Fig. 1. Purification of spinach defensins. A: RP-HPLC fractionation of the 1.5-M LiCl extract from a crude cell-wall preparation from spinach leaves. The gradient used was H_2O (0.1% trifluoroacetic acid)-2-propanol, linear 0–30% for 180 min, 30–50% for 15 min. B: RP-HPLC separation of fractions from A. Same conditions, except that gradient was linear 0–30% for 360 min. C: Separation by SDS-PAGE of the indicated purified proteins. Molecular mass markers (MW) were the multi-colored standard mix from Novex.

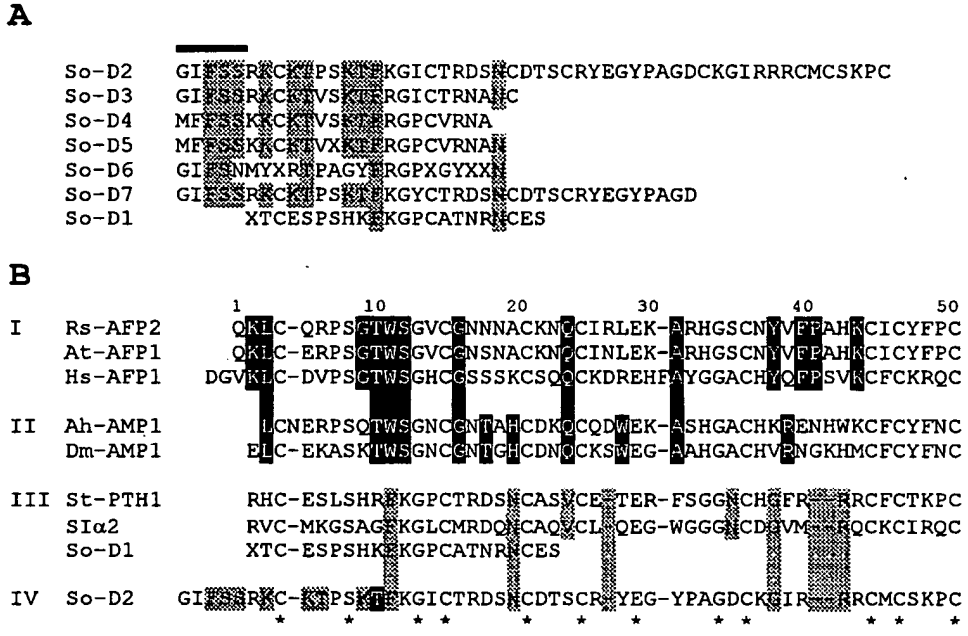

Fig. 2. Alignment of defensin amino acid sequences. A: So-D1-7 defensins. Black horizontal bar indicates the N-terminal extension of group-IV defensins. Highly conserved residues that are relevant for the classification are shaded. B: Comparison of amino acid sequences of defensin groups I–IV. Highly conserved residues that are relevant for the classification (conserved in non-represented, known members of each type) are shaded (black or grey). Residues conserved across all groups are indicated by stars (*). Representative defensin sequences for each group have been taken from the indicated references: Rs-AFP2 [11]; At-AFP1 [14]; Hs-AFP1, Ah-AMP1 and Dm-AMP1 [15]; St-PTH1 [10]; and SIα2 [21].

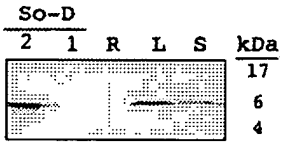

Fig. 3. Western-blot analysis of spinach defensins. So-D2 (1 μg), SO-D1 (1 μg) and total protein extracts from 50 mg of fresh tissue from roots (R), leaves (L) and stems (S). Quantitation by densitometry of Western-blot bands indicates concentrations of defensins of 3 μmol/kg fresh leaves and 1 μmol/kg fresh stems.

3.3. Antimicrobial properties of group-IV defensins

Inhibitory properties of group-IV defensins (So-D2,6,7) were compared with those of two type-III defensins (So-D1; St-PTH1) and of Ta-THα thionin (Table 1). Spinach defensins of groups III and IV were similarly active against the bacteria tested, whereas only those of type IV were active against *Fusarium* spp. Inhibition of *F. culmorum* occurred without hyphal branching and was abolished when salt (1 mM $CaCl_2$ + 50 mM KCl) was added to the medium. Other fungi, such as *Colletotrichum lagenarium* (EC_{50} = 11 μM) and *Bipolaris maydis* (EC_{50} = 6 μM) were also found to be sensitive to So-D2, whereas growth of *Trichodema viridae* and *Septoria nodorum* was not affected at So-D2 concentrations of up to 20 μM.

4. Discussion

We have isolated six new peptides from spinach (So-D2-7) that represent a novel defensin subfamily, as well as one (So-D1) which belongs to the previously described group III of defensins [15]. The new group is structurally closer to group III than to the other two groups, but shows significant divergence with respect to group III at the N-terminal half of the molecule. Of particular interest is a 5-residue N-terminal extension (GIFSS in So-D2) that is present in group-IV and absent in group-III defensins. An unrelated amphibian defensin, esculentin from *Rana esculentum*, has the sequence GIFS at the N terminus [23] and a similar difference is shown by two groups of brevinins, the defensins from *Rana brevipoda porsa* [24]; i.e. brevinins 1 and 2 lack an extension that is present in brevinins 1E and 2E (GLLDSLKG and GIMDTLKN, respectively).

Inhibitory properties of group-IV defensins are summarized and compared with those of the other groups in Table 2. Members of this subfamily are characterized by their ability to inhibit the test fungus *F. culmorum*, without causing multiple budding and swelling of germ tubes and hyphae, as well as both Gram-positive (*Clavibacter michiganensis*) and Gram-negative (*Ralstonia solanacearum*) bacterial pathogens. This means that group-IV defensins resemble those of group II in their antifungal activity and those of group III in their antibacterial activity. The evolution of this peptide family seems to be congruent with its defense role, as the observed structural and functional divergence could have been driven, at least in part, by different challenges represented by the main pathogens of the different plant species. The coexistence of defensins belonging to different subfamilies in the same tissue,

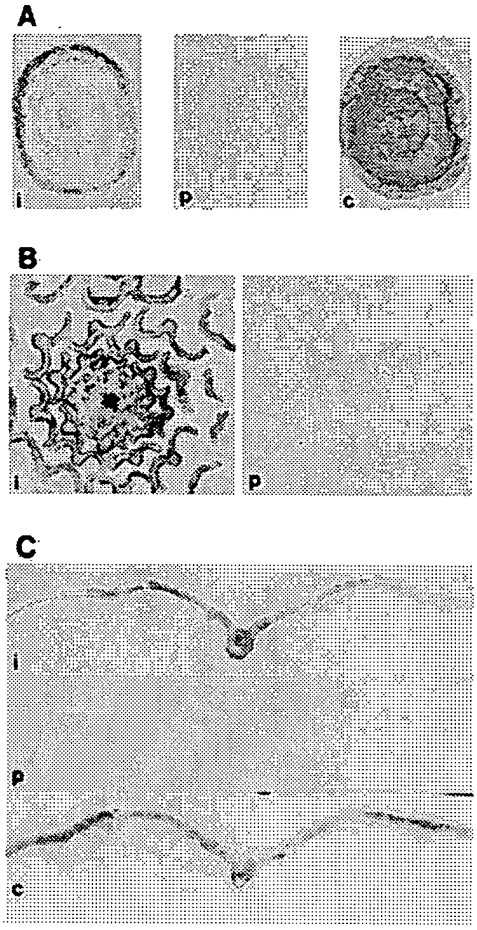

Fig. 4. Tissue-print localization of group-IV defensins from spinach. A: Stem; B: young leaves; C: older leaves. Equivalent sections were stained with immune serum (i), preimmune serum (p) and amido black (c).

Table 1
Inhibition of bacterial and fungal plant pathogens by spinach defensins (So-D1,2,6,7), potato defensin (St-PTH1) and wheat thionin (Ta-THα)

Pathogen	Protein (EC_{50}, μM)[a]					
	So-D				St-PTH	Ta-TH
	1	2	6	7	1	α
Bacteria						
C. michiganensis	1	1	1	0.1	0.2	1
R. solanacearum	15	2	6	1	3	1
Fungi						
F. culmorum	NA	0.2	–	–	NA	0.3
F. solani	NA	11	11	9	7	10
Trichodema viridae	NA	NA	–	–	NA	5

[a]EC_{50} = effective concentration for 50% inhibition; NA, not active at concentrations < 20 μM.

Table 2
Inhibitory properties of defensin subfamilies

Pathogen type	Subfamily			
	I[a]	II[a]	III[a]	IV
Bacteria				
Gram+ ($EC_{50} < 20$ μM)	+	−	+	+
Gram− ($EC_{50} < 20$ μM)	−	−	+	+
Fungus				
F.culmorum ($EC_{50} < 20$ μM)	+	+	−	+
Hyphal branching	+	−	−	−

[a]See [8] and Table 1.

as is reported here, represents a way to achieve a broader antimicrobial barrier in that tissue.

Structure-activity relationships have been investigated in the type-I defensin Rs-AFP2 by site-directed mutagenesis [8]. It is to be noted that, out of 11 positions at which a mutational change produced a significant decrease in the activity of this peptide, only two are conserved in So-D2, namely a T at position 10 and a P at position 50. Furthermore, the change Y→G at position 38 inactivated Rs-AFP2, whereas a G is at that position in So-D2. All these differences would be consistent with the hypothesis of Broekaert and coworkers [1,8], which postulates the existence of more than one mechanism of action among the different defensin groups. Thus, residues that are essential for the activity of group-I defensins would not be necessarily required for activity in groups II and IV.

Acknowledgements: We are indebted to G. Nye (Novartis) and Dr. F. Madueño for their help with MALDI mass spectrometry and amino acid sequencing and to G. Lopez, D. Lamoneda and J. García for techinical assistance. This work was supported by Grant no. PB92-0325 (Dirección General de Investigación Científica y Técnica).

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DOCUMENT-IDENTIFIER: US 6855865 B2
TITLE: Nucleic acids encoding plant defensins and methods of use thereof

Detailed Description Text (5):
By "plant defensin genes", is intended genes that are structurally related to plant defensins, and include thionins, small cysteine-rich peptides, proteinase inhibitors, amylase inhibitors, and the like. They are called defensin genes after a structural classification of proteins (SCOP) classification system. Defensins play a role in defense, more specifically plant defense against pathogens, and they share similarity in primary and secondary structure with insect defensins. While not bound by any mechanism of action, expression of the sequences and related genes around disease-induced lesions may control symptom development, as in a hypersensitive response (HR), by controlling the protease-mediated cell death mechanism. The compositions may also function directly as antipathogenic proteins by inhibiting proteases produced by pathogens or by binding cell wall components of pathogens. Thirdly, they may also act as amphipathic proteins that perturb membrane function, leading to cellular toxicity of the pathogens. These small cysteine-rich peptides demonstrate antimicrobial activity. By "antimicrobial" or "antimicrobial activity" is intended antibacterial, antiviral, antinematocidal, insecticidal, and antifungal activity. Accordingly, the polypeptides of the invention may enhance resistance to insects and nematodes. Any one defensin exhibits a spectrum of antimicrobial activity that may involve one or more antibacterial, antifungal, antiviral, insecticidal, antinematocidal, or antipathogenic activities. They may also be useful in regulating seed storage protein turnover and metabolism.

Detailed Description Text (227):
Inclusion bodies from Example 9 were resuspended in 6 M Guanidine hydrochloride, 0.1 M Tris-HCl, pH 8.0, 1 mM EDTA, and 0.1 M dithiothreitol. After shaking for two hours at low speed on an orbital shaker at room temperature, any remaining particulate matter was removed by centrifugation or filtration.

Other Reference Publication (19):
Thevissen, K., et al., "Fungal Membrane Responses Induced by Plant Defensins and Thionins," J. of Biol. Chem., 1996, pp. 15018-15025, vol. 271(25), The American Society of Biochemistry and Molecular Biology, Inc., USA.

Other Reference Publication (20):
Yamada, S., et al., "cDNA Cloning of.gamma.-Thionin from Nicotiana excelsion," Plant Phys., 1997, p. 314, vol. 115.

transgenic tobacco expressing a barley .alpha.-thionin has increased resistance to Pseudomonas bacterial pathogens (Carmona et al, 1993, Plant J, 3(3):457-462); transgenic tobacco expressing a bean chitinase has increased resistance to the fungal pathogen Rhizoctonia solani (Broglie et al, 1991, Science, 254:1194-1197).

The Ac-AMPs are potent inhibitors of all fourteen fungi tested in this study. Their specificity is comparable to that of wheat thionin which also typically inhibits fungal growth with IC.sub.50 values between 1 and 10 .mu.g/ml (Cammue et al, 1992, J Biol Chem, 267, 2228-2233). Relative to Chitin-binding Plant Proteins, such as the nettle lectin or chitinase, the Ac-AMPs have much higher specific activities. The nettle lectin only inhibits 6 out of 11 fungi at concentrations below 100 .mu.g/ml, whereas at this concentration the pea chitinase is only inhibitory to 1 out of 7 tested fungi.

Detailed Description Text (115):
When added at up to 500 .mu.g/ml to either cultured human umbilical vein endothelial cells or human skin-muscle fibroblasts, neither Ac-AMP1 nor Ac-AMP2 affected cell viability after 24 h of incubation. In contrast, .beta.-purothionin administered at 50 .mu.g/ml decreased the viability of both cell types by more than 90%.

5 entries found for *edta*.

EDTA [P] **Pronunciation Key** (ē'dē-tē-ā')
n.

A crystalline acid, $C_{10}H_{16}N_2O_8$, that acts as a strong chelating agent. The sodium salt of EDTA is used as an antidote for metal poisoning, an anticoagulant, and an ingredient in a variety of industrial reagents.

[e(thylene)d(iamine)t(etraacetic) a(cid).]

Source: *The American Heritage® Dictionary of the English Language, Fourth Edition*

EDTA (ē'dē-tē-ā')
n.

Ethylenediaminetetraacetic acid; a crystalline acid that acts as a strong chelating agent and that forms a sodium salt used as an antidote for metal poisoning and an anticoagulant.

≈104% .05%= 0.0017M
10% = 0.34M
1% = 0.034M
.1% 0.0034M

C 10 × 12 = 120
H 16 × 1 = 16
N 14 × 2 = 28
O 8 × 16 = 128
288

100g/288g = xM/1M
100 = 288(x)

Source: *The American Heritage® Stedman's Medical Dictionary*
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Main Entry: **EDTA**
Pronunciation: "E-"dE-"tE-'A
Function: *noun*
: a white crystalline acid $C_{10}H_{16}N_2O_8$ used especially as a chelating agent and in medicine as an anticoagulant and in the treatment of lead poisoning called also *ethylenediaminetetraacetic acid*

Source: *Merriam-Webster Medical Dictionary, © 2002 Merriam-Webster, Inc.*

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EDTA: in Acronym Finder

Source: *Acronym Finder, © 1988-2004 Mountain Data Systems*

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EDTA: in CancerWEB's On-line Medical Dictionary

Source: *On-line Medical Dictionary, © 1997-98 Academic Medical Publishing & CancerWEB*

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antonyms

Standard periodic table

Group →	1	2		3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
↓ Period																			
1	1 H																		2 He
2	3 Li	4 Be												5 B	6 C	7 N	8 O	9 F	10 Ne
3	11 Na	12 Mg												13 Al	14 Si	15 P	16 S	17 Cl	18 Ar
4	19 K	20 Ca		21 Sc	22 Ti	23 V	24 Cr	25 Mn	26 Fe	27 Co	28 Ni	29 Cu	30 Zn	31 Ga	32 Ge	33 As	34 Se	35 Br	36 Kr
5	37 Rb	38 Sr		39 Y	40 Zr	41 Nb	42 Mo	43 Tc	44 Ru	45 Rh	46 Pd	47 Ag	48 Cd	49 In	50 Sn	51 Sb	52 Te	53 I	54 Xe
6	55 Cs	56 Ba	*	71 Lu	72 Hf	73 Ta	74 W	75 Re	76 Os	77 Ir	78 Pt	79 Au	80 Hg	81 Tl	82 Pb	83 Bi	84 Po	85 At	86 Rn
7	87 Fr	88 Ra	**	103 Lr	104 Rf	105 Db	106 Sg	107 Bh	108 Hs	109 Mt	110 Ds	111 Rg	112 Uub	113 Uut	114 Uuq	115 Uup	116 Uuh	117 Uus	118 Uuo

* Lanthanides	57 La	58 Ce	59 Pr	60 Nd	61 Pm	62 Sm	63 Eu	64 Gd	65 Tb	66 Dy	67 Ho	68 Er	69 Tm	70 Yb
** Actinides	89 Ac	90 Th	91 Pa	92 U	93 Np	94 Pu	95 Am	96 Cm	97 Bk	98 Cf	99 Es	100 Fm	101 Md	102 No

Chemical Series of the Periodic Table

Alkali metals Alkaline earth metals Lanthanides Actinides Transition metals

Poor metals Metalloids Nonmetals Halogens Noble gases

WEST Search History

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DATE: Monday, February 28, 2005

Hide?	Set Name	Query	Hit Count
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☐	L2	L1 and mm	1
☐	L3	$purothionin.clm.	4
☐	L4	l3 and edta	2
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END OF SEARCH HISTORY

THE JOURNAL OF BIOLOGICAL CHEMISTRY
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Intracellular Thionins of Barley

A SECOND GROUP OF LEAF THIONINS CLOSELY RELATED TO BUT DISTINCT FROM CELL WALL-BOUND THIONINS*

(Received for publication, October 13, 1988)

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Leaf thionins of barley have been identified as a novel class of cell wall proteins, toxic to plant pathogenic fungi, and possibly involved in the defense mechanism of plants (Bohlmann, H., Clausen, S., Behnke, S., Giese, H., Hiller, C., Reimann-Philipp, U., Schrader, G., Barkholt, V., and Apel, K., (1988) *EMBO J.* 7, 1559–1565). In the present work a second subfraction of thionins has been detected within the leaf cell, mainly in the vacuole. Thionins of both groups are closely related to each other. They are toxic to phytopathogenic fungi as well as to plant protoplasts, they share similar amino acid sequences, and their synthesis in etiolated seedlings of barley is down-regulated by light. Despite these similarities each of the two subfractions of thionins could be clearly distinguished by its subcellular distribution. In ultrathin sections of embedded etiolated leaf material, cell wall thionins could be immunogold labeled specifically by an antiserum raised against a fusion protein of *Escherichia coli* β-galactosidase and the 15,000 M_r precursor polypeptide of thionins. This antiserum did not react with intracellular thionins. Inversely, intracellular thionins were recognized specifically by an antiserum raised against soluble leaf thionins. The possible function of intracellular thionins as part of a defense mechanism has been discussed.

Plants contain a variety of constitutive and inducible mechanisms for protecting themselves against infections by viruses, bacteria, and fungi (1, 2). Included among these defense mechanisms are the accumulation of low molecular weight lipophilic compounds such as constitutive inhibitins (3) and inducible phytoalexins (4), the reinforcement of the cell wall through the synthesis of lignin-like materials (5), or the deposition of callose (1, 6). Also included are certain proteins, *e.g.* peroxidases (7), proteinase inhibitors (8), chitinases, and β-1, 3-glucanases (9–12), and hydroxyproline-rich glycoproteins (13) that accumulate to high concentrations in plants in response to infections and other stresses. In most cases there is no direct evidence that these substances actually inhibit growth of a pathogen in an infected plant, but their inducibility and *in vitro* activities make this an attractive possibility.

Recently we have detected leaf thionins, a novel class of highly abundant low molecular weight polypeptides with antifungal activity, which are present in cell walls of barley leaves (14, 15). The toxicity of these thionins for plant pathogenic fungi, their localization, and the fact that their synthesis can be triggered by pathogens strongly suggested to us that cell wall thionins are plant proteins possibly involved in the mechanism of plant defense against microbial infection (15, 16). In the present report we describe a closely related second subfraction of thionins which is localized within the leaf cell mainly in the vacuole. The possible function of these intracellular thionins as part of a defense mechanism will be discussed.

EXPERIMENTAL PROCEDURES

Materials—Barley (*Hordeum vulgare* L., cv. Carina) plants were grown in the dark on moist vermiculite at 25 °C for 5 days. Plants were illuminated for different lengths of time as indicated in the figure legends with white light (fluorescent tubes, 8000 lux). Experiments with dark-grown plants were carried out under a dim green safelight. Most of the chemicals used were from Merck. $Na^{35}SO_4$ (0.9–1.5 TBq/mg) was obtained from Amersham Buchler (Braunschweig). A Bio-Rad high performance hydroxylapatite column (100 × 7.8-mm) was used for HPLC.[1] Ion exchange chromatography was carried out on carboxymethyl-cellulose CM-22 (Whatman).

Purification of Thionins—Intracellular, soluble thionins were isolated from total leaf homogenates. 10 g of etiolated barley leaf material were ground under liquid nitrogen. The powder was suspended in 40 ml of 50 mM Na phosphate, pH 7.0, and stirred for 30 min on ice. After centrifugation for 10 min at 10,000 rpm, 3.5 g of ammonium sulfate were dissolved in 10 ml of supernatant. The mixture was placed on ice for 30 min and centrifuged to remove the precipitate. The supernatant was mixed with 1.5 g of ammonium sulfate and stirred on ice for 30 min. For HPLC, the resulting precipitate was dissolved in 45 mM Na phosphate, pH 7.0, and applied to a high performance hydroxylapatite column. The column was pre-equilibrated with 45 mM Na phosphate, pH 7.0, 10 mM $CaCl_2$. Elution occurred with a linear gradient of 45–350 mM Na phosphate, pH 7.0, 10 mM $CaCl_2$. Fractions containing thionin were collected and used for the toxicity tests and for the protein sequencing. For the immunization procedure, final purification was done by preparative SDS-PAGE on a 12–20% linear polyacrylamide gradient gel. Cell wall thionins were isolated according to Bohlmann *et al.* (15). Hordothionins were extracted from barley flour as described by Garcia-Olmedo *et al.* (17) and purified by ion exchange chromatography according to Mak and Jones (18). Intracellular and cell wall thionins of barley

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‖ To whom reprint requests should be addressed.

[1] The abbreviations used are: HPLC, high performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

leaves were reduced and carboxymethylated before they were used for the production of antisera.

Protein Sequencing—Proteins were sequenced by the "Normal-1" procedures on an Applied Biosystems model A 477 sequenator with on-line identification of phenylthiohydantoin-derivatives.

In Vivo Pulse Labeling—For pulse labeling, leaves were cut directly above the seed. After cutting off the basal 5-mm segment under water, 4 leaves were placed in a reaction tube containing 25 μCi of $^{35}SO_4^{2-}$ in 100 μl of water for 4 h in the dark or 2 h in the light. The material was frozen in liquid nitrogen and stored at −20 °C. Alternatively, plants were grown on water culture and $^{35}SO_4^{2-}$ was added to the medium. Both procedures produced the same results.

Toxicity Test—*Thielaviopsis paradoxa* was grown at 28 °C in Petri dishes containing 0.5% potato dextrose agar. Prior to inoculation at the center of the plate, wells punched into the agar were filled with 60 μl of protein solution containing cell wall thionins, soluble thionins, or bovine serum albumin. Protein solutions were applied at a concentration of 500 μM. After 3 days of incubation the fungus had reached the edge of the Petri dish and began to sporulate. At this time the plate was photographed.

Mesophyll protoplasts of *Nicotiana tabacum* cv. Samsun were isolated according to Binding *et al.* (19). After washing, protoplasts at a concentration of 5×10^4/ml were suspended in 1 ml of 3% agarose which contained different concentrations of soluble or cell wall thionins ranging from 0 to 20 μM and were kept in small Petri dishes for up to 2 weeks at 20 °C under dim light until callus formation in thionin-free control samples was visible. In controls, bovine serum albumin was added to the agarose at the same concentrations as thionins. No effect of bovine serum albumin on callus formation could be detected.

Immunological Detection of Proteins—Proteins were separated on SDS-polyacrylamide gels according to Laemmli (20). If not stated otherwise, proteins were reduced with β-mercaptoethanol and carboxymethylated prior to electrophoresis. Proteins were transferred electrophoretically from polyacrylamide gels to nitrocellulose sheets according to Towbin *et al.* (21). Thionins were detected immunologically on nitrocellulose sheets as described by Dehesh and Ryberg (22). Immunogold labeling of thionins in ultrathin sections of etiolated barley leaves was done as described by Dehesh *et al.* (23).

Isolation of Vacuoles and Miniprotoplasts from Mesophyll Protoplasts of Barley—Mesophyll protoplasts from barley primary leaves were prepared as described by Kaiser *et al.* (24). Vacuoles were liberated from the protoplasts by mechanical lysis (25). Contamination with other cell constituents was less than 1% (26). Miniprotoplasts were liberated from protoplasts by the evacuolation technique described by Lörz *et al.* (27) which has been slightly modified. 2 ml of a suspension of purified protoplasts were layered on 10 ml of a solution containing 400 mM mannitol, 50 mM $CaCl_2$, 10 mM Hepes-imidazol, pH 7.0, dissolved in Percoll, and were centrifuged for 30 min at 120,000 × *g*. The resulting miniprotoplasts lack the central vacuole but contain more than 85% of the other cell organelles and 30–50% of the cytosol.[2]

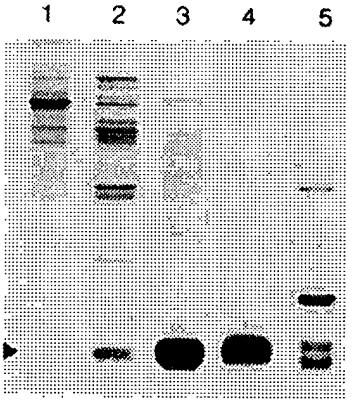

FIG. 1. **Isolation and purification of putative soluble thionins from total leaf homogenates of dark-grown barley (*H. vulgare*) seedlings.** Soluble proteins of leaf homogenates (*1*), after precipitation between 35 and 50% (w/v) ammonium sulfate (*2*) and after HPLC (*3*) were separated electrophoretically on a 15–20% linear polyacrylamide gradient gel together with the putative soluble thionins purified by preparative gel electrophoresis (*4*). *5*, standard proteins: chymotrypsinogen (25,000), cytochrome *c* (12,500), trypsin inhibitor (6,500), and hordothionin (5,000). *Arrowhead* indicates the position of the putative soluble thionin.

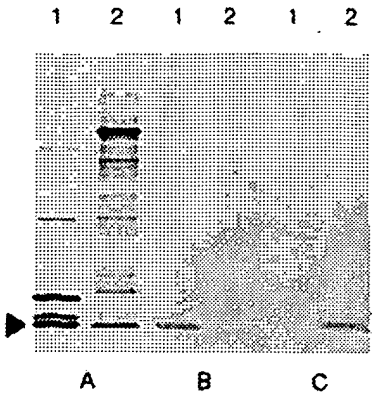

FIG. 2. **Immunological relationship between hordothionin and the putative soluble leaf thionin of barley.** *1*, standard proteins including hordothionin (*arrowhead*) and *2*, soluble leaf proteins were separated electrophoretically on a 12–20% polyacrylamide gradient gel. After separation, proteins were stained with Coomassie Blue (*A*) or blotted onto nitrocellulose and detected immunologically using the antisera against hordothionin (*B*) or against the putative soluble leaf thionin (*C*). Standard proteins: ovalbumin (45,000), chymotrypsinogen (25,000), cytochrome *c* (12,500), trypsin inhibitor (6,500), and hordothionin (5,000). Proteins were reduced with β-mercaptoethanol and carboxymethylated prior to electrophoresis.

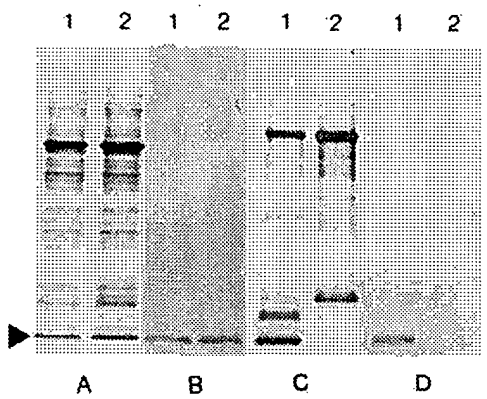

FIG. 3. **The effect of light on the synthesis of soluble putative thionins in leaves of barley seedlings kept in the dark or illuminated for 12 h with white light prior to incubation.** $Na^{35}SO_4$ was fed to detached leaves of either etiolated barley seedlings in the dark (*1*) or pre-illuminated seedlings in the light (*2*), as described under "Experimental Procedures." Soluble leaf proteins were separated electrophoretically on a 12–20% polyacrylamide gradient gel. Proteins were stained with Coomassie Blue (*A*) or blotted onto nitrocellulose and detected immunologically using the antiserum against soluble leaf thionins (*B*). *C* and *D*, demonstration of the light-induced decline in the synthesis of the putative soluble leaf thionin. *C*, autoradiogram of the gel shown in *A*. *D*, immunoprecipitate of radioactively labeled soluble leaf proteins with an antiserum raised against the putative soluble thionin.

RESULTS

Isolation of Soluble Thionins from Barley Leaves—In the past we have noticed that in etiolated barley seedlings there is a highly abundant mRNA encoding a 15,000 M_r polypeptide whose concentration rapidly declines upon illumination (28, 29). This mRNA is present at high concentrations in the leaf and mesocotyl of etiolated barley seedlings but almost undetectable in other tissues of the plants like roots or the endosperm (30). The 15,000 M_r polypeptide has been identified as

[2] E. Martinoia and G. Kaiser, unpublished results.

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                                10                  20                  30                  40          46
Leaf Thionin BTH6   K S C C K D T L A K N C Y N T C R F A G G S R P V C A G A C R C K I I S G P K C P S D Y P K

Cell Wall Thionin   ? ? ? ? * * * * * * * ? * * * ? ? * * * * * g * * ? * * * ? ? ? * * *
                              Q V       L   F   I                       A       V

Soluble Thionin     * * ? ? * * * * * * * ? * * * ? ? r *
                              N V   G
```

FIG. 4. **A comparison of the amino acid sequence of barley leaf thionin as predicted from the nucleotide sequence of the thionin gene of the genomic DNA clone BTH6 (15) with the partial amino acid sequences of cell wall and soluble thionins purified as described under "Experimental Procedures."** The partial amino acid sequences are shown below that of BTH6 with only the amino acid differences indicated. Cysteine residues had not been determined. The *question marks* in the protein sequence indicate residues where no clear signal was obtained during the sequence analysis. At some positions more than 1 amino acid was found indicating that the fractions analyzed contain more than one sequence variant of thionin.

a high molecular weight precursor polypeptide of leaf thionins which is composed of a signal peptide, a thionin domain, and an acidic polypeptide (14, 31). Two different experimental approaches have been used to identify and to localize thionins within the leaf of barley seedlings.

In our previous work an antiserum was raised against a fusion protein of *Escherichia coli* β-galactosidase and the 15,000 M_r precursor polypeptide of leaf thionins. The antiserum against the isolated fusion protein was allowed to react with ultrathin sections of embedded leaf material of etiolated barley seedlings. After the samples had been processed with protein A-gold particles, the antigens were found exclusively in the cell wall. Electrophoretic analysis of proteins extracted from isolated cell walls revealed that thionins represent a major subfraction of cell wall proteins of barley leaves (15).

In the present work putative thionin-like proteins were isolated from total leaf extracts. The soluble proteins of the leaf homogenate were separated electrophoretically on SDS-polyacrylamide gels. A low molecular weight protein was present among the soluble leaf proteins whose apparent molecular weight was very similar to that of the 5,000 M_r hordothionin of barley seeds (Fig. 1). This protein could be purified further by ammonium sulfate precipitation. Among the proteins which precipitated between 35 and 50% (w/v) ammonium sulfate, this protein was one of the most abundant polypeptides (Fig. 1). It could be purified to apparent homogeneity through HPLC on hydroxylapatite columns and subsequent preparative gel electrophoresis (Fig. 1).

An antiserum was raised against the reduced and carboxymethylated putative thionin-like protein and was used to determine the relationship of this leaf protein with the thionin of barley seeds. Equal amounts of hordothionin and the 5000 M_r leaf protein were reduced and carboxymethylated, separated electrophoretically, transferred onto nitrocellulose, and allowed to react with the antisera against hordothionin or the putative thionin-like leaf protein, respectively. The antiserum raised against the leaf protein reacted strongly with this protein, but cross-reacted also with the hordothionin. The antiserum raised against hordothionin reacted in an inverse way with the two polypeptides (Fig. 2).

The immunological cross-reactivity and the similar size of the two polypeptides suggested to us that the low molecular weight polypeptide extracted from leaf homogenates could be a soluble thionin. This hypothesis was tested by analyzing the effect of light on the biosynthesis of the leaf protein. It had been shown previously that the concentration of mRNAs encoding leaf thionins rapidly declines once the etiolated barley seedling has been exposed to light (28, 29). Etiolated seedlings were kept in the dark or illuminated with white light for 12 h. Primary leaves were detached from these seedlings and placed in a solution containing $Na^{35}SO_4$. After incubation either in the dark or in the light, proteins were extracted from the leaves and separated electrophoretically on a SDS 12–20%-polyacrylamide gradient gel. An aliquot of each protein fraction was incubated with the antiserum against the putative thionin-like protein and the resulting immunoprecipitate applied to the polyacrylamide gel. The distribution of radioactively labeled polypeptides was determined by autoradiography. In leaves of etiolated barley seedlings the 5000 M_r polypeptide was heavily labeled with ^{35}S while in leaves of illuminated seedlings the extent of labeling of this protein had been drastically reduced (Fig. 3). This result is in agreement with the effect of light on the concentration of thionin-specific mRNAs. The identity of the polypeptide as thionin was confirmed by partial sequence determination of the isolated soluble 5000 M_r leaf protein. The partial sequence of this polypeptide is closely related to both the amino acid sequence of cell wall thionins as well as the corresponding

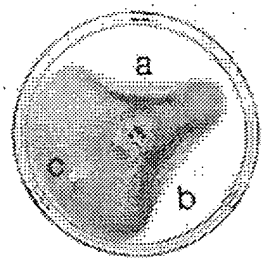
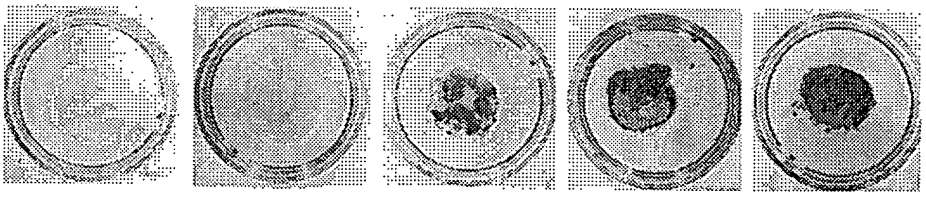


FIG. 5. **Growth inhibition of the phytopathogenic fungus *T. paradoxa* by cell wall (*a*) and soluble (*b*) thionins of barley leaves.** *c*, bovine serum albumin taken as a control did not affect the growth of the fungus.

FIG. 6. **The effect of different concentrations of soluble thionins on callus formation from protoplasts of *N. tabacum*.** *Numbers* indicate the various thionin concentrations in micromolars. Comparable results were obtained with cell wall thionins. Bovine serum albumin did not affect callus formation.



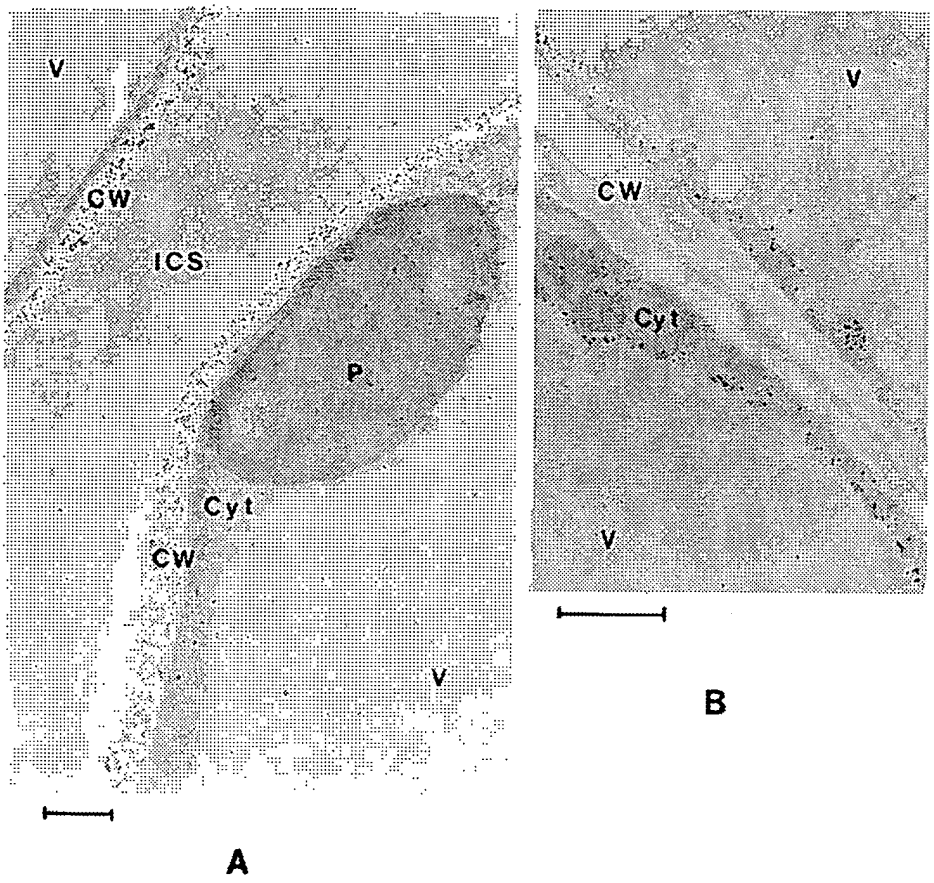


FIG. 7. **Localization of intracellular and cell wall thionins in leaves of dark-grown barley.** Sections of Lowicryl-embedded tissue slices were prepared and allowed to react with the antisera directed against the fusion protein (*A*) and the soluble thionins (*B*). Subsequently they were treated with 20 nm protein A-gold as described by Dehesh *et al.* (23). Comparison of the results clearly demonstrates that intracellular thionins are recognized specifically by the antiserum against the soluble thionins while cell wall thionins react only with the antibody against the fusion protein. *CW*, cell wall; *Cyt*, cytoplasm; *P*, plastid; *V*, vacuole; *ICS*, intercellular space. *Bar* = 0.5 μm.

part of the thionin precursor as derived from the nucleotide sequence of a thionin gene (Fig. 4).

The Toxicity of Soluble Thionins—Most thionins studied so far including the cell wall thionins of barley leaves have been shown to be highly toxic for various bacteria, fungi, and small animals (15, 32–34). The toxicity of the soluble thionin was tested using the fungus *T. paradoxa*, a pathogen of sugar cane (35). Soluble and cell wall thionins were applied at a concentration of 500 μM each to the test plates. Both proteins were able to suppress the growth of the fungus, while bovine serum albumin as a control protein had no detectable effect on the fungus if applied at the same concentration (Fig. 5).

Thionins have not been found in the intercellular fluid of barley leaves (36), thus, the soluble thionins isolated from leaf homogenates appear to be present within the plant cell. In such a case it is important to know whether or not intracellular thionins are toxic to plant cells. Thionins are assumed to disrupt the permeability of cell membranes (34, 37, 38). The toxicity of thionins for plant cells was tested by exposing isolated tobacco mesophyll protoplasts to increasing concentrations of soluble and cell wall thionins isolated from barley leaves. Protoplasts were kept in 3% agarose for at least 2 weeks to allow callus formation. As shown in Fig. 6 thionin concentrations as low as 5×10^{-6} M damaged protoplasts such that callus formation did not occur. Soluble and cell wall thionins were effective in inhibiting callus formation at roughly the same concentration. Bovine serum albumin added to the protoplasts at the same concentration as thionins did not affect the callus formation (results not shown).

The Localization of Soluble Thionins in Barley Leaves—In our previous work leaf thionins had been localized within the cell wall of barley leaves (15). This was demonstrated in two different ways. First, by immunogold labeling of ultrathin

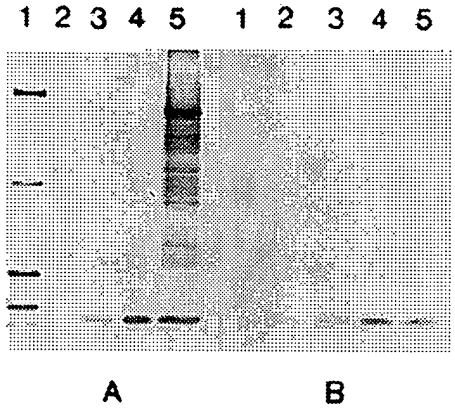


FIG. 8. **Comparison of the relative concentrations of cell wall (2–4) and soluble (5) leaf thionins in etiolated barley seedlings.** Soluble and cell wall proteins were extracted from the same amount of leaf tissue and separated electrophoretically on a 15–20% linear polyacrylamide gradient gel. In *lane 5* proteins applied to the gel correspond to 13 mg of leaf, fresh weight; in *lanes 2–4* they correspond to 147, 294, and 588 mg of leaf, fresh weight, respectively. After separation, proteins were stained in the gel with Coomassie Blue (*A*) or blotted onto nitrocellulose and detected immunologically using the antiserum against soluble leaf thionins (*B*). *1*, Standard proteins: bovine serum albumin (68,000), chymotrypsinogen (25,000), cytochrome *c* (12,500), trypsin inhibitor (6,500), and hordothionin (5,000). Proteins were reduced with β-mercaptoethanol and carboxymethylated prior to electrophoresis.

sections of leaves using an antiserum against a fusion protein of *E. coli* β-galactosidase and the 15,000 M_r precursor polypeptide of thionins. Second, by extracting thionins from isolated cell walls. Cell walls had been sonicated and washed extensively with water prior to the extraction without any apparent loss of wall-bound thionins. Cell wall thionins could

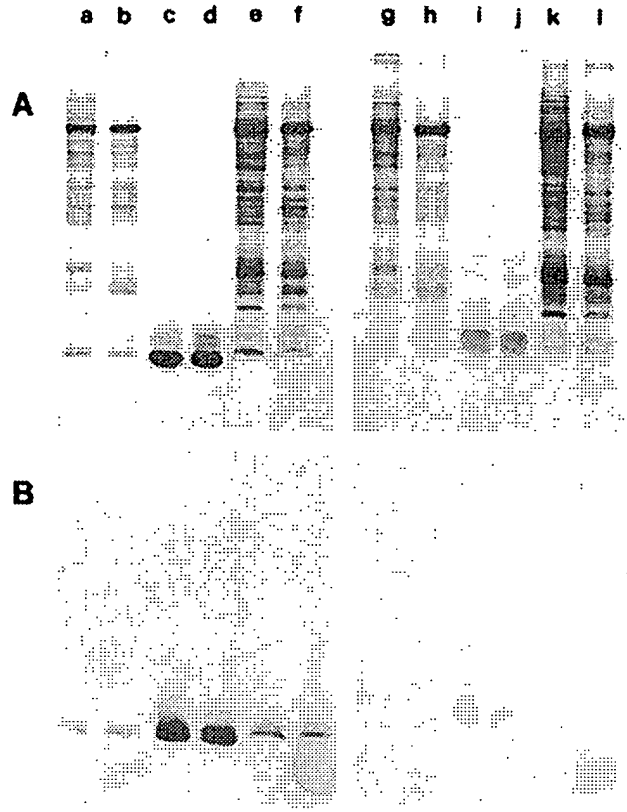


FIG. 9. **The effect of reduced and carboxymethylated sulfhydryl groups of leaf thionins on their immunoreactivity.** Soluble leaf proteins (*a, b, g, h*), cell wall proteins (*c, d, i, j*), and total leaf proteins extracted in the presence of SDS from leaf homogenates (*e, f, k, l*) were isolated from leaves of dark-grown (*a, c, e, g, i, k*) or 12-h illuminated (*b, d, f, h, j, l*) barley seedlings. The various protein fractions were solubilized in the presence of SDS and applied to 15–20% linear polyacrylamide gradient gels without any further treatment (*g–l*) or treated with β-mercaptoethanol and carboxymethylated prior to electrophoresis (*a–f*). After separation, proteins were stained with Coomassie Blue (*A*) or blotted onto nitrocellulose and detected immunologically using the antiserum against the soluble thionin.

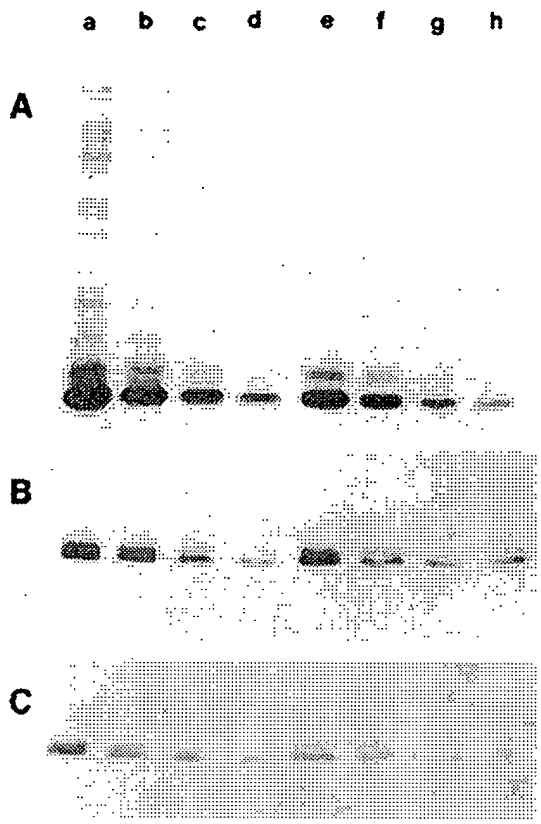


FIG. 10. **Comparison of the immunoreactivity of cell wall and soluble thionins.** Increasing amounts of cell wall (*a–d*) and soluble thionins (*e–h*), ranging 2.5 (*d, h*), 5 (*c, g*), 10 (*b, f*), and 20 (*a, e*) μg, were applied to a 15–20% polyacrylamide gradient gel. Thionins were treated with β-mercaptoethanol and carboxymethylated prior to electrophoresis. After separation, proteins were stained with Coomassie Blue (*A*) or blotted onto nitrocellulose and detected immunologically using antisera against the fusion protein (*B*) or the soluble thionins (*C*).

be separated from the cell wall only after the addition of high concentrations of salt. Furthermore, thionins could be localized by immunogold labeling only outside the cell within the wall (15). These findings seem to be in conflict with results obtained in the present work, which indicate that thionins are a major subfraction of soluble intracellular leaf proteins.

With the antiserum against the fusion protein, the results of the immunogold labeling were similar to those reported earlier (Fig. 7*A*). Immunoreactive proteins were detected only within the area of the cell wall. When ultrathin sections of the same leaf sample were allowed to react with the antiserum against the soluble thionin, a completely different picture emerged from the immunogold labeling. Gold particles were absent from the cell wall and could be seen only within the cell mainly in the area of the tonoplast (Fig. 7*B*). The density of gold particles within the cell was significantly lower than in the cell wall. This result could suggest that intracellular thionins are present in the leaf at a much lower concentration than cell wall thionins. However, comparison of the amounts of soluble thionins and cell wall thionins, which could be isolated from the same quantity of etiolated leaf material, revealed that soluble thionins comprise an approximately 40-fold higher portion of total leaf thionins than cell wall thionins (Fig. 8). As shown previously by immunogold labeling of isolated cell walls before and after extraction with LiCl, solubilization of cell wall thionins was almost complete (15). Thus, the small portion of cell wall thionins relative to the amount of soluble thionins was not due to an insufficient extraction procedure.

The specificities of the two antisera used for the immunogold labeling were compared further by allowing the antisera to react with the two isolated subfractions of leaf thionins after these thionins had been treated with SDS. Thionins were either reduced with β-mercaptoethanol and carboxymethylated to block reactive sulfhydryl groups prior to electrophoretic separation or were analyzed without any further treatment. Nonreduced thionins were scarcely recognized by the two antisera but the thionins reacted very strongly with the antisera after they had been reduced and carboxymethylated (Fig. 9). A dilution series of equal amounts of the purified reduced and carboxymethylated soluble and cell wall thionins was separated electrophoretically on a SDS 15–20%-polyacrylamide gradient gel and was transferred onto nitrocellulose. There was no longer any apparent difference in the immunoreactivity of the two different subfractions of thionins after the polypeptides had been reduced and carboxymethylated and blotted onto nitrocellulose in the presence of SDS (Fig. 10).

We have tried to explain the apparent discrepancy between the results of immunogold labeling of thionins in ultrathin sections of etiolated barley leaves and the comparison of quantities of soluble and cell wall thionins which could be extracted from the same amount of leaf material. Mesophyll

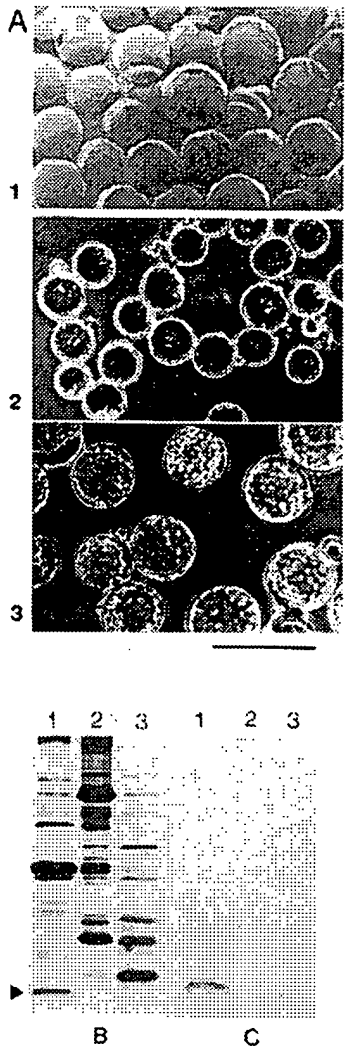


FIG. 11. **The intracellular distribution of thionins in isolated mesophyll protoplasts of barley.** Protoplasts were isolated from leaves of barley seedlings which had been exposed to continuous white light for 48 h. Vacuoles (*1*) and miniprotoplasts (*2*) which lack the central vacuole were liberated from protoplasts (*3*) as described under "Experimental Procedures." *A*, microscopic pictures of the various cellular and subcellular fractions. *B* and *C*, the distribution of thionins among these fractions. Proteins were reduced with β-mercaptoethanol, carboxymethylated, and separated electrophoretically on a SDS 15–20% linear polyacrylamide gradient gel. Proteins were stained in the gel with Coomassie Blue (*B*) or blotted onto nitrocellulose and detected immunologically using an antiserum against soluble leaf thionins (*C*). Almost all of the intracellular thionins are present within the central vacuole. *Arrowhead* indicates the position of the intracellular thionin. *Bar* = 50 μm.

protoplasts were prepared from leaves of barley seedlings which had been grown in the dark before they were exposed to continuous white light for 48 h. Vacuoles and miniprotoplasts were liberated from the protoplasts (Fig. 11*A*). Miniprotoplasts lack the central vacuole but contain more than 85% of the other cell organelles and 30–50% of the cytosol (26). Contamination of the vacuole preparation with other cell constituents was less than 1% (26). Equal amounts of proteins of the various cellular and subcellular fractions were applied to a 15–20% polyacrylamide gradient gel after they had been reduced and carboxymethylated. Proteins were separated electrophoretically and either stained in the gel with Coomassie Blue or allowed to react with the antiserum against the soluble thionins. Large amounts of thionins were present in vacuoles but thionins could not have been detected within the fraction of miniprotoplasts (Fig. 11, *B* and *C*). Thus, a major part of intracellular thionins is stored within the central vacuole of the leaf cells. Probably these proteins have been lost during the preparation of ultrathin sections of barley leaves and thus could not have been detected by immunogold labeling.

DISCUSSION

Recently, leaf thionins of barley have been identified as a novel class of cell wall proteins (14, 15). In the present work a second subfraction of thionins has been described which is localized within the leaf cell. Thionins of both groups are closely related to each other. They are toxic to phytopathogenic fungi as well as to protoplasts of tobacco, they share similar amino acid sequences and their synthesis in etiolated seedlings of barley is down-regulated by light.

Despite these similarities each of the two subfractions of thionins could be clearly distinguished by its subcellular distribution. In ultrathin sections of embedded etiolated leaf material, cell wall thionins could be immunogold labeled specifically by an antiserum raised against a fusion protein of *E. coli* β-galactosidase and the 15,000 M_r precursor polypeptide of thionins. This antiserum did not react with the intracellular thionins of these leaf sections. On the other hand, an antiserum raised against soluble thionins did not immunogold label cell wall thionins. The extent of immunogold labeling of intracellular thionins by the latter antiserum seemed to indicate that this subgroup of thionins makes up only a minor fraction of total thionins of barley leaves. Analysis of the soluble polypeptides extracted from leaf homogenates of etiolated seedlings, however, revealed that the amounts of soluble thionins/g of leaf, fresh weight, exceed by far those of the cell wall thionins. Comparison of the thionin content of isolated vacuoles and miniprotoplasts, which had been liberated from mesophyll protoplasts of barley leaves revealed that the major portion of intracellular thionins is stored within the central vacuole of leaf cells. We assume that these thionins had been lost during the preparation of ultrathin sections of barley leaves. In such a case, intracellular thionins which had been detected by immunogold labeling in the vicinity of the tonoplast would represent only a minor subfraction of total intracellular thionins.

The immunoreactivity of thionins is strongly affected by the folding of the polypeptide. When blotted onto nitrocellulose, thionins of the two subfractions reacted with the two antisera only when they had been reduced and carboxymethylated prior to the electrophoretic separation. On the other hand, the immunogold labeling of thionins in ultrathin sections of embedded barley leaf material revealed that each of the two subgroups reacted only with one of the two sera. These latter results indicate that thionins of different subcellular compartments must differ from each other in their exposed antigenic sites. For instance, in ultrathin sections cell wall thionins were recognized only by the antiserum against the fusion protein. Within the fusion protein the basic thionin part of the precursor molecule is linked to the acidic protein. It is likely that both parts interact with each other such that new epitopes of thionins are being exposed which normally are hidden within the mature thionin polypeptide. The results of immunogold labeling of cell wall thionins suggest that at least some of these epitopes are also exposed in thionins which have been deposited within the cell wall.

Thionins are assumed to disrupt the permeability of cell membranes (34, 37, 38). In this respect they resemble other toxins such as the killer toxin of yeast (39), the cecropins of

insects (40), or the perforin of cytotoxic T lymphocytes (41). Cells that produce and store these toxins must be immune to their action. All thionins whose mRNA sequences have been studied so far are synthesized as a high molecular weight precursor molecule which contains an acidic polypeptide part, in addition to the basic thionin part. We believe that the acidic part of the precursor polypeptide might neutralize a toxic effect of the thionin within the cell. The charge differences between the basic thionin and the acidic protein part would favor a close interaction of both protein domains. Since cysteine residues have been highly conserved not only within different thionin variants but also within the corresponding acidic protein parts one could easily visualize that thionins could be inactivated within the precursor molecule through intramolecular disulfide bonds between the thionin and the acid protein domains.

Even though there is no experimental clue available so far as to what function the intracellular thionins might have, they are so closely related to cell wall thionins that it seems likely that they probably function in a very similar way as the cell wall thionins. Thus, an attractive possibility is that these thionins are also part of a defense mechanism. We are currently trying to test this proposed role of intracellular thionins in barley plants.

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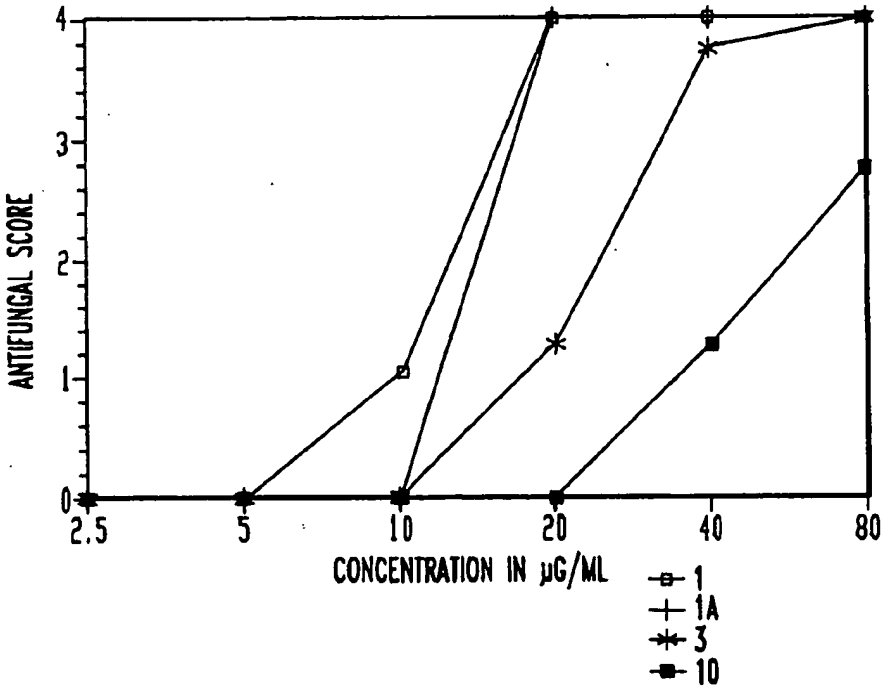

(57) Abstract

Derivatives of α-hordothionin made by position-specific substitution with lysine residues provide lysine enrichment while retaining the antifungal activity of the parent compound.

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HIGH LYSINE DERIVATIVES OF ALPHA-HORDOTHIONIN

TECHNICAL FIELD

This invention relates to derivatives of α-hordothionin which provide higher percentages of lysine while retaining the antifungal functionality of hordothionins.

BACKGROUND OF THE INVENTION

Disease resistance is an important objective of the genetic engineering of crop plants. Numerous fungi and bacteria are serious pests of common agricultural crops. One method of controlling diseases has been to apply antimicrobial organic or semiorganic chemicals to crops. This method has numerous, art-recognized problems. A more recent method of control of microorganism pests has been the use of biological control organisms which are typically natural competitors or inhibitors of the troublesome microorganisms. However, it is difficult to apply biological control organisms to large areas, and even more difficult to cause those living organisms to remain in the treated area for an extended period. Still more recently, techniques in recombinant DNA have provided the opportunity to insert into plant cells cloned genes which express antimicrobial compounds. This technology has given rise to additional concerns about eventual microbial resistance to well-known, naturally occurring antimicrobials, particularly in the face of heavy selection pressure, which may occur in some areas. Thus, a continuing effort is underway to express naturally occurring antimicrobial compounds in plant cells directly by translation of a single structural gene.

However, the use of such techniques gives rise to further problems. Crop plants have more important things to do than fight disease. They are sources of sugars, starches, proteins, oils, fibers, and other raw materials. Genetic

engineers would also like to modify, and often to enhance, the production of those natural plant products. Unfortunately, plant cells can only produce large quantities of a few cellular components at a time. If they are producing high levels of storage proteins, it is difficult for them to also produce high levels of antifungal compounds. Thus, genetic engineers face a quandary in designing advanced plant systems with existing molecules for protein quality enhancement and disease resistance which require concurrent high-level expression of multiple genes.

Brief Description of the Drawing Figures

Figure 1 is a graph of antimicrobial performance of various compounds discussed herein against S. sclerotiorum.

Figure 2 is a graph of antimicrobial performance of various compounds discussed herein against A. flavus.

Figure 3 is a graph of antimicrobial performance of various compounds discussed herein against F. graminearum.

Figure 4 is a graph of antimicrobial performance of various compounds discussed herein against F. moniliforme.

DISCLOSURE OF THE INVENTION

It has now been determined that one class of compounds, the α-hordothionins, can be modified to enhance their content of lysine while maintaining their antifungal activity. These hordothionin derivatives can be expressed to simultaneously enhance both resistance to fungal diseases and lysine content of the plant.

α-hordothionin is a 45-amino acid protein which has been well characterized. It can be isolated from seeds of barley (Hordeum vulgare) and even in its native form is especially rich in arginine and lysine residues, containing 5 residues (10%) of each. The amino acid sequence is as provided in SEQUENCE I.D. No. 1. It has powerful antifungal properties. Initial work to enhance the lysine content of this protein provided a high lysine derivative as indicated in SEQUENCE

I.D. No. 2. However, it was impossible to predict the ultimate effect of this seemingly trivial substitution on the tertiary structure and folding of the protein, and subsequent bioassays determined that this derivative did not fold to a biologically active species in vitro. In addition, both tertiary structure and folding are critical to the stability and adequate expression of the protein in vivo, and both were absent in this compound. Therefore, further analysis and functional modeling of the wild-type compound was undertaken to determine whether substitutions could be made without disrupting biological activity. Although the crystal structure of crambin, a small protein of similar size and structure, has been reported, such crystal structures have not previously been available for hordothionin or even related compounds such as purothionin and viscotoxin. We undertook to develop such structural information.

Three-dimensional modeling of the protein led us to believe that the arginine residue at position 10 was critical to retention of the appropriate 3-dimensional structure and possible folding through hydrogen bond interactions with the C-terminal residue of the protein. A lysine substitution at that point with its shorter side chains could not hydrogen bond at the same time to both the serine residue at the 2 position and to the C-terminus while maintaining the backbone structure which we had predicted. The synthetic peptide having this substitution could not be made to fold correctly, which supported this analysis. Conservation of the arginine residue at position 10 provided a protein which folded correctly, had the sequence indicated in SEQUENCE I.D. No. 3, and exhibited antifungal activity in a bioassay. Comparison of the structure of hordothionin with that of the loosely related (48% homologous, 30% identical) protein crambin showed that thionin had a disulfide bond linking the cysteines at positions 12 and 29 which was not bridging the corresponding positions in crambin. Accordingly, replacement of the cysteine at position 12 of thionin with lysine and replacement of the cysteine at position 29 with threonine to produce a protein having the sequence indicated in SEQUENCE I.D. No. 4

was found not to disrupt the 3-dimensional structure of the protein, as evidenced by an energy content which was determined to be indistinguishable from that of the native protein.

Further analysis of substitutions which would not alter the 3-dimensional structure of the molecule led to replacement of Asparagine-11, Glutamine-22 and Threonine-41 with lysine residues with virtually no steric hindrance. The resulting compound had the sequence indicated in SEQUENCE I.D. No. 5, containing 29% lysine residues. In addition, it was determined that by replacement of the serine residue at position 2 with aspartic acid, the arginine at position 10 could be replaced with lysine while permitting the needed hydrogen bonding with the C-terminus, providing a compound of the sequence indicated in SEQUENCE I.D. No. 6. It should be appreciated that that these substitutions would be effective and acceptable could not have been predicted by examination of the linear sequence of the native thionin-protein.

Other combinations of these substitutions were also made, providing proteins having the sequences indicated in sequence i.d. no. 7 and sequence i.d. no. 8. accordingly, this invention provides proteins having the sequence of sequence i.d. no. 9 wherein the amino acid residues at one or more of positions 5, 10, 11, 12, 17, 19, 22, 30 and 41 are lysine, and the remainder of the residues at those positions are the residues at the corresponding positions in sequence i.d. no. 1, provided that the residue at position 30 is threonine when the residue at position 12 is lysine and cysteine otherwise, and the residue at position 2 is aspartic acid when the residue at position 10 is lysine and serine otherwise. although the native hordothionin is relatively lysine rich, a storage protein with 10% lysine residues (by number) cannot be expressed at high enough levels to obtain total protein lysine contents which are sufficient to obviate the need for lysine supplementation in poultry and swine feeds. these compounds are significantly more lysine enriched, and can be made to contain nearly thirty percent lysine residues. without such enhanced lysine contents, it is impossible to eliminate the

need for lysine supplementation of feeds. this invention thus also provides an important method for enhancing the lysine content of a plant cell or a plant, comprising the step of causing one or more proteins according to this invention to be expressed in the cell or plant.

synthesis of the compounds was performed according to methods of peptide synthesis which are well known in the art and thus constitute no part of this invention. in vitro, we have synthesized the compounds on an applied biosystems model 431a peptide synthesizer using fastmoc^{tm} chemistry involving hbtu [2-(1h-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate, as published by rao et al., int. j. pep. prot. res. 40:508-515 (1992). peptides were cleaved following standard protocols and purified by reverse phase chromatography using standard methods. the amino acid sequence of each peptide was confirmed by automated edman degradation on an applied biosystems 477a protein sequencer/120a pth analyzer. more preferably, however, the compounds of this invention are synthesized in vivo by bacterial or plant cells which have been transformed by insertion of an expression cassette containing a synthetic gene which when transcribed and translated yields the desired compound. such empty expression cassettes, providing appropriate regulatory sequences for plant or bacterial expression of the desired sequence, are also well-known, and the nucleotide sequence for the synthetic gene, either rna or dna, can readily be derived from the amino acid sequence for the protein using standard reference texts. Preferably, such synthetic genes will employ plant-preferred codons to enhance expression of the desired protein.

Industrial Applicability

The following description further exemplifies the compositions of this invention and the methods of making and using them. However, it will be understood that other methods, known by those of ordinary skill in the art to be equivalent, can also be employed.

Plants

The polypeptides employed in this invention can be effectively applied to plants afflicted with susceptible microorganisms by any convenient means, including spray, creams, dust or other formulation common to the antimicrobial arts. The compound can also be incorporated systemically into the tissues of a treated plant so that in the course of infesting the plant the pathogens will be exposed to antimicrobial amounts of the compound of this invention. One method of doing this is to incorporate the compound in a non-phytotoxic vehicle which is adapted for systemic administration to the susceptible plants. This method is commonly employed with fungicidal materials such as captan and is well within the purview of one of ordinary skill in the art of plant fungicide formulation. However, since the genes which code for these compounds can be inserted into an appropriate expression cassette and introduced into cells of a susceptible plant species, an especially preferred embodiment of this method involves inserting into the genome of the plant a DNA sequence coding for a compound of this invention in proper reading frame, together with transcription initiator and promoter sequences active in the plant. Transcription and translation of the dna sequence under control of the regulatory sequences causes expression of the protein sequence at levels which provide an antimicrobial amount of the protein in the tissues of the plant which are normally infected by the pathogens.

the plant is preferably a plant susceptible to infection and damage by one or more of fusarium graminearum, fusarium moniliforme, aspergillus flavus, alternaria longipes, sclerotinia sclerotiorum, and sclerotinia trifoliorum. these include corn (zea mays) and sorghum (sorghum bicolor). however, this is not to be construed as limiting, inasmuch as these two species are among the most difficult commercial crops to reliably transform and regenerate, and these pathogens also infect certain other crops. thus the methods of this invention are readily applicable via conventional techniques to numerous plant species, if they are found to be

susceptible to the plant pathogens listed hereinabove, including, without limitation, species from the genera allium, antirrhinum, arabidopsis, arachis, asparagus, atropa, avena, beta, brassica, browallia, capsicum, cicer, cicla, citrullus, citrus, cucumis, cucurbita, datura daucus, digitalis, fagopyrum, fragaria, geranium, glycine, gossypium, helianthus, hordeum, hemerocallis, lactuca, lens, lolium, lotus, lycopersicon, majorana, manihot, medicago, nasturtium, nicotiana, oryza, pelargonium, persea, petunia, phaseolus, pisum, ranunculus, raphanus, ricinus, saccharum, secale, senecio, setaria, solanum, spinacia, trifolium, triticum, bromus, cichorium, hyoscyamus, linum, nemesia, panicum, onobrychis, pennisetum, salpiglossis, sinapis, trigonella, and vigna.

preferred plants that are to be transformed according to the methods of this invention are cereal crops, including maize, rye, barley, wheat, sorghum, oats, millet, rice, triticale, sunflower, alfalfa, rapeseed and soybean.

synthetic dna sequences can then be prepared which code for the appropriate sequence of amino acids, and this synthetic dna sequence can be inserted into an appropriate plant expression cassette.

likewise, numerous plant expression cassettes and vectors are well known in the art. by the term "expression cassette" is meant a complete set of control sequences including initiation, promoter and termination sequences which function in a plant cell when they flank a structural gene in the proper reading frame. expression cassettes frequently and preferably contain an assortment of restriction sites suitable for cleavage and insertion of any desired structural gene. it is important that the cloned gene have a start codon in the correct reading frame for the structural sequence. in addition, the plant expression cassette preferably includes a strong constitutive promoter sequence at one end to cause the gene to be transcribed at a high frequency, and a poly-a recognition sequence at the other end for proper processing and transport of the messenger rna. an example of such a preferred (empty) expression cassette into which the cdna of

the present invention can be inserted is the pphi414 plasmid developed by beach et al. of pioneer hi-bred international, inc., johnston, ia, as disclosed in u.s. patent application no. 07/785,648, filed october 31, 1991. highly preferred plant expression cassettes will be designed to include one or more selectable marker genes, such as kanamycin resistance or herbicide tolerance genes.

by the term "vector" herein is meant a dna sequence which is able to replicate and express a foreign gene in a host cell. typically, the vector has one or more endonuclease recognition sites which may be cut in a predictable fashion by use of the appropriate enzyme. such vectors are preferably constructed to include additional structural gene sequences imparting antibiotic or herbicide resistance, which then serve as markers to identify and separate transformed cells. preferred markers/selection agents include kanamycin, chlorosulfuron, phosphonothricin, hygromycin and methotrexate. a cell in which the foreign genetic material in a vector is functionally expressed has been "transformed" by the vector and is referred to as a "transformant."

a particularly preferred vector is a plasmid, by which is meant a circular double-stranded dna molecule which is not a part of the chromosomes of the cell.

as mentioned above, both genomic and cdna encoding the gene of interest may be used in this invention. the vector of interest may also be constructed partially from a cdna clone and partially from a genomic clone. when the gene of interest has been isolated, genetic constructs are made which contain the necessary regulatory sequences to provide for efficient expression of the gene in the host cell. according to this invention, the genetic construct will contain (a) a first genetic sequence coding for the protein or trait of interest and (b) one or more regulatory sequences operably linked on either side of the structural gene of interest. typically, the regulatory sequences will be selected from the group comprising of promoters and terminators. the regulatory sequences may be from autologous or heterologous sources.

promoters that may be used in the genetic sequence include nos, ocs and camv promoters.

an efficient plant promoter that may be used is an overproducing plant promoter. overproducing plant promoters that may be used in this invention include the promoter of the small sub-unit (ss) of the ribulose-1,5-biphosphate carboxylase from soybean (berry-lowe et al., j. molecular and app. gen., 1:483-498 (1982)), and the promoter of the cholorophyll a-b binding protein. these two promoters are known to be light-induced, in eukaryotic plant cells (see, for example, genetic engineering of plants, an agricultural perspective, a. cashmore, pelham, new york, 1983, pp. 29-38, g. coruzzi et al., j. biol. chem., 258:1399 (1983), and p. dunsmuir, et al., j. molecular and app. gen., 2:285 (1983)).

the expression cassette comprising the structural gene for the protein of this invention operably linked to the desired control sequences can be ligated into a suitable cloning vector. in general, plasmid or viral (bacteriophage) vectors containing replication and control sequences derived from species compatible with the host cell are used. the cloning vector will typically carry a replication origin, as well as specific genes that are capable of providing phenotypic selection markers in transformed host cells. typically, genes conferring resistance to antibiotics or selected herbicides are used. after the genetic material is introduced into the target cells, successfully transformed cells and/or colonies of cells can be isolated by selection on the basis of these markers.

typically, an intermediate host cell will be used in the practice of this invention to increase the copy number of the cloning vector. with an increased copy number, the vector containing the gene of interest can be isolated in significant quantities for introduction into the desired plant cells. host cells that can be used in the practice of this invention include prokaryotes, including bacterial hosts such as e. coli, s. typhimurium, and serratia marcescens. eukaryotic hosts such as yeast or filamentous fungi may also be used in this invention. since these hosts are also microorganisms, it

will be essential to ensure that plant promoters which do not cause expression of the protein in bacteria are used in the vector.

the isolated cloning vector will then be introduced into the plant cell using any convenient technique, including electroporation (in protoplasts), retroviruses, bombardment, and microinjection into cells from monocotyledonous or dicotyledonous plants in cell or tissue culture to provide transformed plant cells containing as foreign dna at least one copy of the dna sequence of the plant expression cassette. preferably, the monocotyledonous species will be selected from maize, sorghum, wheat or rice, and the dicotyledonous species will be selected from soybean, alfalfa, rapeseed, sunflower or tomato. using known techniques, protoplasts can be regenerated and cell or tissue culture can be regenerated to form whole fertile plants which carry and express the gene for a protein according to this invention. accordingly, a highly preferred embodiment of the present invention is a transformed maize plant, the cells of which contain as foreign dna at least one copy of the dna sequence of an expression cassette of this invention.

finally, this invention provides methods of imparting resistance to diseases caused by microorganisms selected from fusarium graminearum, fusarium moniliforme, diplodia maydis, colletototrichum graminicola, verticillium alboatrum, phytophthora megaspermae f.sp. glycinea, macrophomina phaseolina, diaporthe phaseolorum caulivora, sclerotinia sclerotiorum, sclerotinia trifoliorum, aspergillus flavus to plants of a susceptible taxon, comprising the steps of:

a) culturing cells or tissues from at least one plant from the taxon,

b) introducing into the cells or tissue culture at least one copy of an expression cassette comprising a structural gene for one or more of the compounds of this invention, operably linked to plant regulatory sequences which cause the expression of the compound or compounds in the cells, and

c) regenerating disease-resistant whole plants from the cell or tissue culture. once whole plants have been obtained,

they can be sexually or clonally reproduced in such manner that at least one copy of the sequence provided by the expression cassette is present in the cells of progeny of the reproduction.

alternatively, once a single transformed plant has been obtained by the foregoing recombinant dna method, conventional plant breeding methods can be used to transfer the structural gene for the compound of this invention and associated regulatory sequences via crossing and backcrossing. such intermediate methods will comprise the further steps of

a) sexually crossing the disease-resistant plant with a plant from the disease-susceptible taxon;

b) recovering reproductive material from the progeny of the cross; and

c) growing disease-resistant plants from the reproductive material. where desirable or necessary, the agronomic characteristics of the susceptible taxon can be substantially preserved by expanding this method to include the further steps of repetitively:

a) backcrossing the disease-resistant progeny with disease-susceptible plants from the susceptible taxon; and

b) selecting for expression of antimicrobial activity (or an associated marker gene) among the progeny of the backcross, until the desired percentage of the characteristics of the susceptible taxon are present in the progeny along with the gene imparting antimicrobial activity.

by the term "taxon" herein is meant a unit of botanical classification of genus or lower. it thus includes genus, species, cultivars, varieties, variants, and other minor taxonomic groups which lack a consistent nomenclature.

it will also be appreciated by those of ordinary skill that the plant vectors provided herein can be incorporated into agrobacterium tumefaciens, which can then be used to transfer the vector into susceptible plant cells, primarily from dicotyledonous species. thus, this invention provides a method for imparting antimicrobial activity and disease resistance in agrobacterium tumefaciens-susceptible dicotyledonous plants in which the expression cassette is

introduced into the cells by infecting the cells with agrobacterium tumefaciens, a plasmid of which has been modified to include a plant expression cassette of this invention.

Human and veterinary pharmaceutical use

This invention also provides methods of treating and preventing infection by susceptible organisms in a human or lower animal host in need of such treatment, which method comprises administration to the human or lower animal host in need of such treatment a therapeutically effective amount of a polypeptide of this invention or a composition containing one or more of the polypeptides. The polypeptides of the present invention may be administered parenterally, by inhalation spray, rectally or topically in dosage unit formulations containing conventional nontoxic pharmaceutically acceptable carriers, adjuvants and vehicles as desired. The term parenteral as used herein includes subcutaneous, intravenous, intramuscular, intraarticular and intrathecal injection and infusion techniques. As with other polypeptides, the polypeptides of this invention are not known to be active orally.

Total daily dose of the compounds of this invention administered to a host in single or divided doses may be in amounts, for example, of from 1 to 2000 mg/kg body weight daily and more usually 50 to 500 mg/kg. Dosage unit compositions may contain such amounts or fractions or submultiples thereof as appropriate to make up the daily dose. It will be understood, however, that the specific dose level for any particular patient will depend upon a variety of factors including the activity of the specific compound employed, the age, body weight, general health, sex, diet, time of administration, route of administration, rate of excretion, drug combination and the severity of the particular disease undergoing therapy.

This invention also provides pharmaceutical compositions in unit dosage form, comprising an effective amount of a

compound of this invention in combination with a conventional pharmaceutical carrier. As used herein, the term "pharmaceutical carrier" means a solid or liquid filler, diluent or encapsulating material. Some examples of the materials which can serve as pharmaceutical carriers are sugars, such as lactose, glucose and sucrose; starches such as corn starch and potato starch; cellulose and its derivatives such as sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate; powdered tragacanth; malt; gelatin; talc; excipients such as cocoa butter and suppository waxes; oils such peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; polyols such as propylene glycol, glycerin, sorbitol, mannitol and polyethylene glycol; esters such as ethyl oleate and ethyl laurate; agar; buffering agents such as magnesium hydroxide and aluminum hydroxide; alginic acid; pyrogen-free water; isotonic saline; Ringer's solution, ethyl alcohol and phosphate buffer solutions, as well as other non-toxic compatible substances used in pharmaceutical formulations. Wetting agents, emulsifiers and lubricants such as sodium lauryl sulfate and magnesium stearate, as well as coloring agents, release agents, coating agents, and perfuming agents and preservatives can also be present in the compositions, according to the desires of the formulator. The amount of active ingredient that may be combined with the carrier materials to produce a single dosage form will vary depending upon the host treated and the particular mode of administration.

By "therapeutically effective amount" herein is meant an amount of either polypeptide or combination thereof sufficient to provide antimicrobial activity so as to alleviate or prevent infection by susceptible organisms in the human or lower animal being treated at a reasonable benefit/risk ratio attendant with any medical treatment.

Antifungal Testing

The antifungal activity of compounds synthesized in accord with this invention was measured using art-recognized methods,

as described in Duvick et al., J. Biol. Chem. 26:18814-18820 (1992) against Aspergillus flavus, S. sclerotiorum, Fusarium graminearum and F. moniliforme. Results are shown in Figures 1 through 4.

In Figure 1, the curves are labeled according to their SEQUENCE I.D. Nos., with the exception of the curve labeled "10," which reflects the performance of a crude mixture of correctly and incorrectly folded compounds, including some quantity of the compound having SEQUENCE I.D. No. 2. The two curves labeled "1" and "1a" represent results obtained with natural and synthetic hordothionin, respectively, both having the sequence shown in SEQUENCE I.D. No. 1.

In Figures 2 through 4, the curves labeled as "1" and "1a" again represent results obtained with natural and synthetic hordothionin, respectively, both having the sequence shown in SEQUENCE I.D. No. 1. The curves for other derivatives are labeled according to the SEQUENCE I.D. No. of the derivative.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Rao, A. Gururaj; Beach, Larry

(ii) TITLE OF INVENTION: HIGH LYSINE DERIVATIVES OF α-HORDOTHIONIN

(iii) NUMBER OF SEQUENCES: 9

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Pioneer Hi-Bred International, Inc.

(B) STREET: 700 Capital Square, 400 Locust Street

(C) CITY: Des Moines

(D) STATE: Iowa

(E) COUNTRY: United States

(F) ZIP: 50309

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Diskette, 3.5 inch, 1.44 Mb storage

(B) COMPUTER: IBM Compatible

(C) OPERATING SYSTEM: MS-DOS, Microsoft Windows

(D) SOFTWARE: Microsoft Windows Notepad

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE:

(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Roth, Michael J.

(B) REGISTRATION NUMBER: 29,342

(C) REFERENCE/DOCKET NUMBER: 0233 US

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (515) 245-3594

(B) TELEFAX: (515) 245-3634

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 45 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: protein
(A) DESCRIPTION: hordothionin
(iii) HYPOTHETICAL: No
(iv) ANTI-SENSE: NO
(v) FRAGMENT TYPE :
(vi) ORIGINAL SOURCE:
(A) ORGANISM: Hordeum vulgare
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Lys Ser Cys Cys Arg Ser Thr Leu Gly Arg
5 10
Asn Cys Tyr Asn Leu Cys Arg Val Arg Gly
15 20
Ala Gln Lys Leu Cys Ala Gly Val Cys Arg
25 30
Cys Lys Leu Thr Ser Ser Gly Lys Cys Pro
35 40
Thr Gly Phe Pro Lys
45

(2) INFORMATION FOR SEQ ID NO: 2:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 45 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: protein
(A) DESCRIPTION: hordothionin derivative
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Lys Ser Cys Cys Lys Ser Thr Leu Gly Lys
5 10
Asn Cys Tyr Asn Leu Cys Lys Val Lys Gly
15 20
Ala Gln Lys Leu Cys Ala Gly Val Cys Lys
25 30
Cys Lys Leu Thr Ser Ser Gly Lys Cys Pro
35 40

Thr Gly Phe Pro Lys
45

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 45 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(A) DESCRIPTION: hordothionin derivative

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Lys Ser Cys Cys Lys Ser Thr Leu Gly Arg
5 10

Asn Cys Tyr Asn Leu Cys Lys Val Lys Gly
15 20

Ala Gln Lys Leu Cys Ala Gly Val Cys Lys
25 30

Cys Lys Leu Thr Ser Ser Gly Lys Cys Pro
35 40

Thr Gly Phe Pro Lys
45

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 45 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(A) DESCRIPTION: hordothionin derivative

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Lys Ser Cys Cys Lys Ser Thr Leu Gly Arg
5 10

Asn Lys Tyr Asn Leu Cys Lys Val Lys Gly
15 20

Ala Gln Lys Leu Cys Ala Gly Val Thr Lys
25 30

Cys Lys Leu Thr Ser Ser Gly Lys Cys Pro
35 40

Thr Gly Phe Pro Lys
45

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 45 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(A) DESCRIPTION: hordothionin derivative

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Lys Ser Cys Cys Lys Ser Thr Leu Gly Arg
5 10

Lys Lys Tyr Asn Leu Cys Lys Val Lys Gly
15 20

Ala Lys Lys Leu Cys Ala Gly Val Thr Lys
25 30

Cys Lys Leu Thr Ser Ser Gly Lys Cys Pro
35 40

Lys Gly Phe Pro Lys
45

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 45 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(A) DESCRIPTION: hordothionin derivative

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Lys Asp Cys Cys Lys Ser Thr Leu Gly Lys
5 10

Lys Lys Tyr Asn Leu Cys Lys Val Lys Gly
15 20

Ala Lys Lys Leu Cys Ala Gly Val Thr Lys
25 30

Cys Lys Leu Thr Ser Ser Gly Lys Cys Pro
35 40

Lys Gly Phe Pro Lys
45

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 45 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(A) DESCRIPTION: hordothionin derivative

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

Lys Ser Cys Cys Lys Ser Thr Leu Gly Arg
5 10

Lys Cys Tyr Asn Leu Cys Lys Val Lys Gly
15 20

Ala Gln Lys Leu Cys Ala Gly Val Cys Lys
25 30

Cys Lys Leu Thr Ser Ser Gly Lys Cys Pro
35 40

Lys Gly Phe Pro Lys
45

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 45 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(A) DESCRIPTION: hordothionin derivative

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

Lys Ser Cys Cys Lys Ser Thr Leu Gly Arg
5 10

Lys Cys Tyr Asn Leu Cys Lys Val Lys Gly
15 20

Ala Lys Lys Leu Cys Ala Gly Val Cys Lys
25 30

Cys Lys Leu Thr Ser Ser Gly Lys Cys Pro
35 40

Lys Gly Phe Pro Lys
45

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 45 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(A) DESCRIPTION: hordothionin derivative

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

Lys Xaa Cys Cys Xaa Ser Thr Leu Gly Xaa
5 10
Xaa Xaa Tyr Asn Leu Cys Xaa Val Xaa Gly
15 20
Ala Lys Xaa Leu Cys Ala Gly Val Xaa Xaa
25 30
Cys Xaa Leu Thr Ser Ser Gly Xaa Cys Pro
35 40
Thr Gly Phe Pro Xaa
45

WHAT IS CLAIMED IS:

1. A protein having the sequence of SEQUENCE I.D. No. 9 wherein the amino acid residues at one or more of positions 5, 10, 11, 12, 17, 19, 22, 30 and 41 are lysine, and the remainder of the residues at those positions are the residues at the corresponding positions in SEQUENCE I.D. No. 1, provided that the residue at position 30 is threonine when the residue at position 12 is lysine and cysteine otherwise, and the residue at position 2 is aspartic acid when the residue at position 10 is lysine and serine otherwise.

2. A protein according to Claim 1 wherein one or more of the the amino acid residues at positions 5, 11, 12, 17, 19, 22 and 41 are lysine.

3. A protein according to Claim 2 wherein all of the amino acid residues at positions 5, 11, 12, 17, 19, 22 and 41 are lysine.

4. A nucleotide sequence which codes for a protein according to Claim 1.

5. An RNA sequence according to Claim 4.

6. A DNA sequence according to Claim 4.

7. An expression cassette containing the DNA sequence of claim 6 operably linked to plant regulatory sequences which cause the expression of the DNA sequence in plant cells.

8. A bacterial transformation vector comprising an expression cassette according to Claim 7, operably linked to bacterial expression regulatory sequences which cause replication of the expression cassette in bacterial cells.

9. Bacterial cells containing as a foreign plasmid at least one copy of a bacterial transformation vector according to Claim 8.

10. Transformed plant cells containing at least one copy of the expression cassette of Claim 7.

11. Transformed cells according to Claim 10, further characterized in being cells of a monocotyledonous species.

12. Transformed cells according to Claim 11, further characterized in being maize, sorghum, wheat or rice cells.

13. Transformed cells according to Claim 10, further characterized in being cells of a dicotyledonous species.

14. Transformed cells according to Claim 13, further characterized in being soybean, alfalfa, rapeseed, sunflower, tobacco or tomato cells.

15. A maize cell or tissue culture comprising cells according to claim 12.

16. A transformed plant comprising transformed cells according to Claim 10.

17. A method for killing and inhibiting plant pathogenic microorganisms which are susceptible to a-Hordothionin comprising introducing into the environment of the pathogenic microorganisms an antimicrobial amount of a protein according to Claim 1.

18. A method for killing and inhibiting plant pathogens selected from Fusarium graminearum, Fusarium moniliforme, Diplodia maydis, Colletototrichum graminicola, Verticillium alboatrum, Phytophthora megaspermae f.sp. glycinea, Macrophomina phaseolina, Diaporthe phaseolorum caulivora, Sclerotinia sclerotiorum, Sclerotinia trifoliorum, and

Aspergillus flavus, comprising introducing into the environment of the pathogenic microorganisms an antimicrobial amount of a protein according to Claim 1.

19. A method according to Claim 17 wherein the environment of the pathogen is the tissues of a living plant.

20. A method for enhancing the lysine content of a plant cell or seed comprising the step of causing a protein according to Claim 1 to be expressed in the cell or seed.

21. A method for enhancing the lysine content of a plant comprising the step of causing a protein according to claim 1 to be expressed in tissues of the plant.

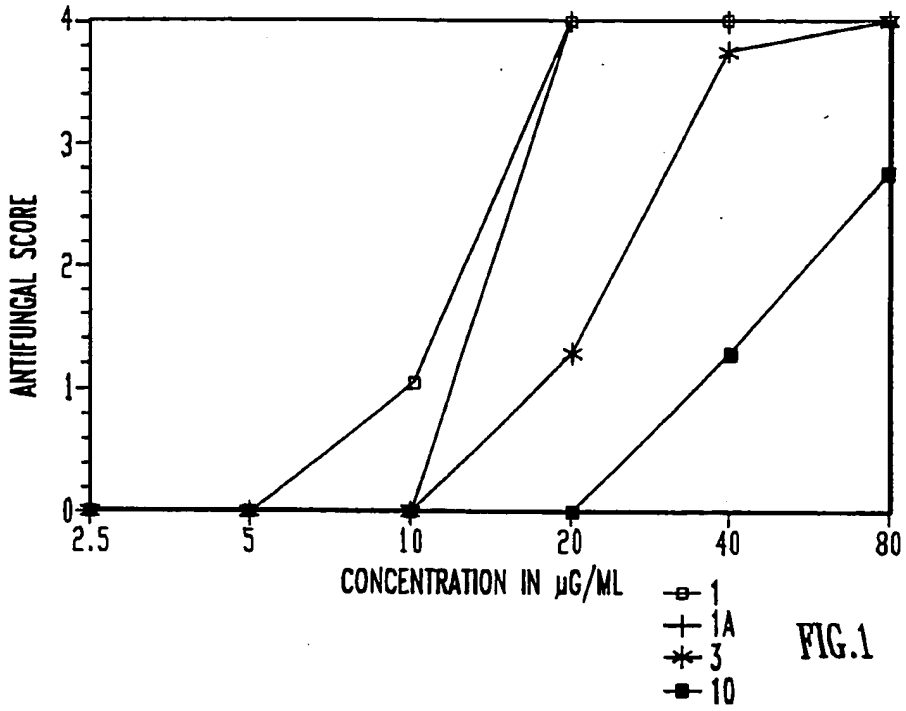
ANTIFUNGAL SCORE
0
1
2
3
4
2.5
5
10
20
40
80
CONCENTRATION IN µG/ML
1
1A
3
10

FIG.1

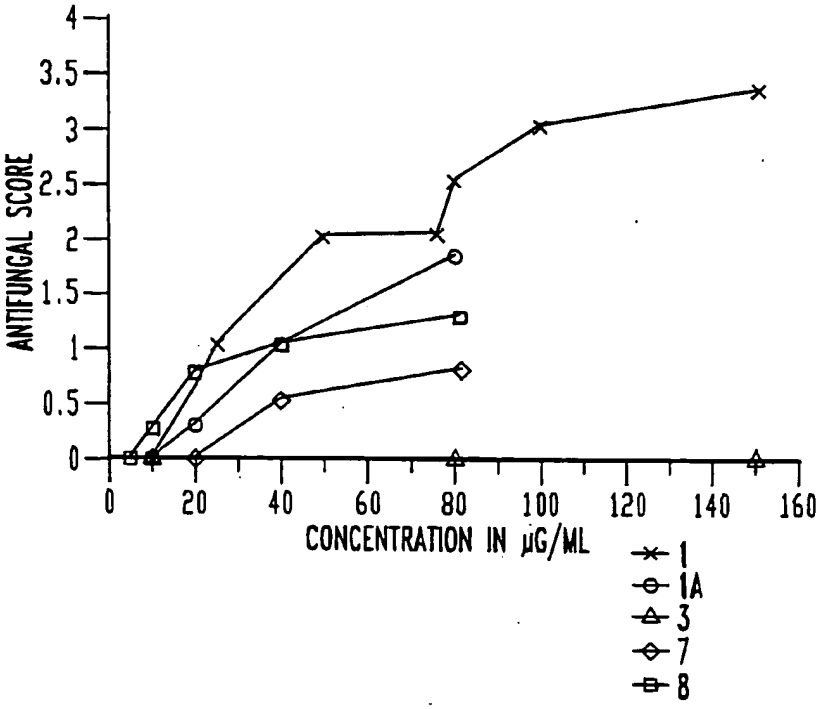
ANTIFUNGAL SCORE
0
0.5
1
1.5
2
2.5
3
3.5
4
0
20
40
60
80
100
120
140
160
CONCENTRATION IN µG/ML
1
1A
3
7
8

FIG.2

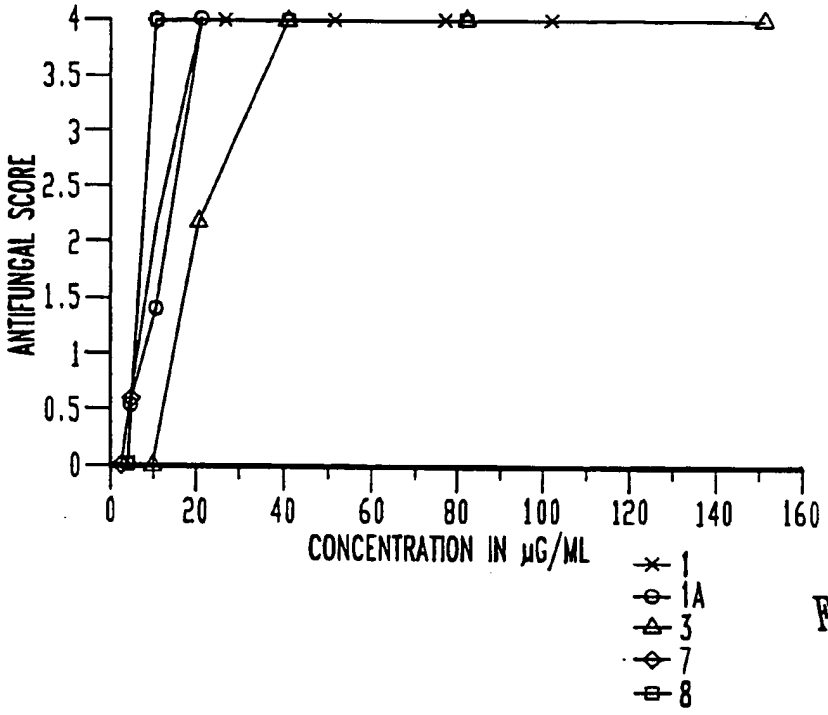
ANTIFUNGAL SCORE
0
0.5
1
1.5
2
2.5
3
3.5
4
0
20
40
60
80
100
120
140
160
CONCENTRATION IN µG/ML
1
1A
3
7
8

FIG.3

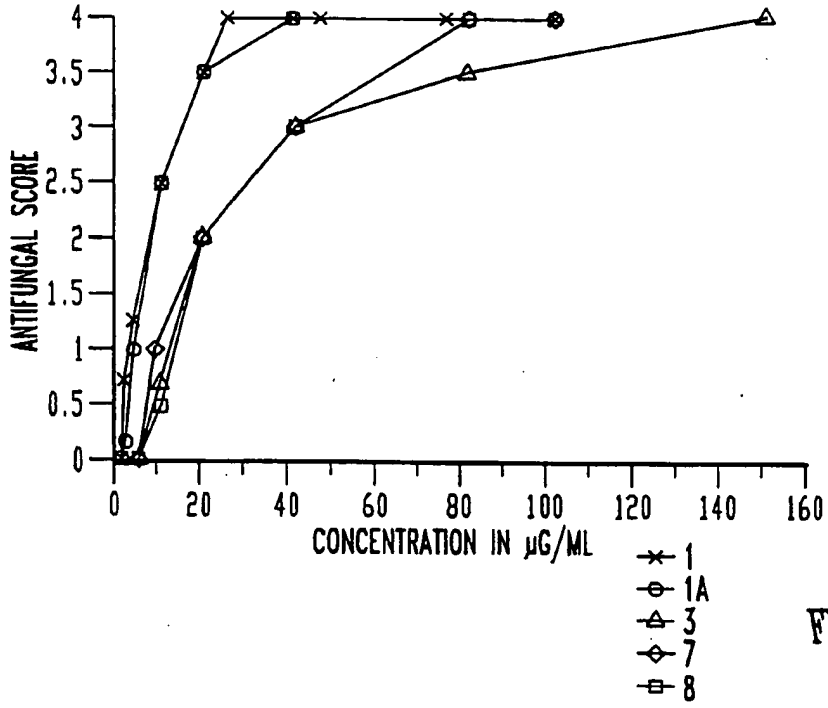
ANTIFUNGAL SCORE
0
0.5
1
1.5
2
2.5
3
3.5
4
0
20
40
60
80
100
120
140
160
CONCENTRATION IN µG/ML
1
1A
3
7
8

FIG.4

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(54) Thionin stain technique.

(57) Cellular analysis in accordance with the invention involves staining cellular material with a thionin stain to distinguish the nuclear portion from the cytoplasm, counterstaining, illuminating the thionin-stained cellular material with energy in the infrared spectrum, and imaging the stained cellular material. The analysis can be automated or manual.

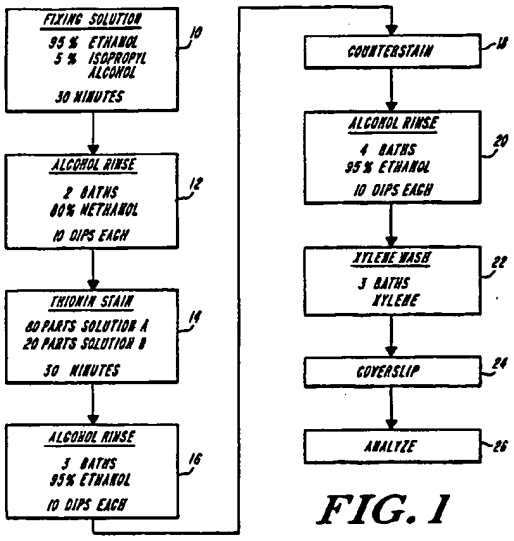

FIG. 1

EP 0 445 434 A2

Field of the Invention

This invention relates to the analysis of cellular material. Specifically, this invention relates to a method of staining cells for cytological or histological analysis to contrast the nuclear portion of the cell from the cytoplasmic portion. The analysis can be automated or manual.

Background of the Invention

A traditional multicolored stain is desirable for staining cell smears for certain cytological analyses. It is advantageous because the nucleus and the cytoplasm of the stained specimen are colored differently. In one preferred staining practice, the cytoplasm is transparent, whereas the nucleus is transparent to opaque. This staining pattern allows the cytologist to distinguish cells which are morphologically abnormal. In addition, cytologists find the variety of colors of the traditional stains, particularly the Papanicolaou stain, helpful to reduce eye strain and to aid diagnosis.

However, the traditional stains, including the Papanicolaou stain, are difficult for an automated system to analyze. The variety of colors in the cytoplasm from traditional stains, which are straightforward for the human eye to distinguish, are not readily analyzed with automated systems because they contrast to varying degrees with the traditional blue hematoxylin stain of the nucleus. The varying contrast makes automated analysis unreliable.

A number of researchers have developed algorithms in an attempt to attain automated analysis of cells stained with the Papanicolaou stain. Most techniques involve the use of various instrumental artifacts, such as different colors of light, filters, and color television cameras. Many require a high level of sophistication that is costly in terms of hardware and software. Further, these approaches have not proven accurate and reliable enough to be widely used in clinical cytological and histological diagnoses.

It is an object of the present invention to provide a method of cellular analysis in which the cells are multicolored and the nuclear portion is clearly distinguishable from the cytoplasmic portion both with automated equipment and with manual vision analysis.

It is a further object to provide a method of cellular analysis in which the characteristics of the stained cells can be accurately determined with both manual and automated analysis procedures.

Other objects of the invention will in part be obvious and will in part appear hereinafter.

Summary of the Invention

The method of cellular analysis in accordance with the invention can be used as part of an automated analysis system and for manual analysis. It entails staining cellular material with a thionin stain that clearly contrasts the nuclear portion from the cytoplasmic portion under infrared illumination. In a further step, the cellular material is counterstained with a stain that absorbs infrared light at a level distinguishable from that of the thionin stain. Another step involves illuminating the stained cellular material with energy in the infrared spectrum, and a further step involves imaging the stained cellular material with the output energy from the interaction of the infrared energy with the stained cellular material.

The improvement of this method over the art includes illuminating the cellular material, previously stained with the thionin stain, with energy in the infrared spectrum. It has now been found that a mildly acidic, organic thionin stain compound can be strongly absorbing in the infrared spectral region when bound to nuclear material. This allows physiological parameters of the individual cell components, i.e., size, configuration and texture, to be distinguished and analyzed under infrared illumination. The imaging step preferably includes detecting the output energy with a light-sensing, opto-electric transducing device which may include photomultipliers, solid-state photo-detectors, detector arrays, and television cameras. The imaging step preferably includes digitizing the transduced image, for computerized analysis and pathological diagnosis of the stained cellular material.

The thionin stain used in the method of the invention consists of a thionin salt, which is preferably thionin acetate, an organic solvent which is preferably a low molecular weight alcohol and more preferably methanol, and a pH buffer. One preferred pH buffer is prepared with glacial acetic acid, distilled water, and 5N NaOH to about pH 4.9.

The thionin stain is used in combination with a counterstain that manifests the cytoplasmic portion under visible light. In one practice of the invention, the cellular material is stained with the thionin compound, rinsed in a low molecular weight alcohol, and then counter-stained with a traditional Papanicolaou stain. In another practice of the invention, the counterstain is an eosin-type stain.

The Papanicolaou stain, when applied to cells stained with the thionin stain, produces the multicolored

cells obtained from staining with the traditional Papanicolaou alone. This particular combination of the thionin stain and the Papanicolaou stain provides a system useful both for human visual analysis and for automated analysis. Further, the automated analysis and characterization of the cell can be carried out without the need for costly hardware and/or software. The cytoplasm can be analyzed under visible light and the nucleus can be analyzed under infrared illumination.

A similar advantage is obtained by staining the cell first with a thionin stain and then with a counterstain which is an eosin-type stain. This thionin-eosin stain replaces the traditional H-E (hematoxalin-eosin) histological stain with one that gives the same visual effect as the H-E stain, and has the added advantage of distinguishing the nucleus from the cytoplasm under infrared light. Incorporating the method of the present invention into conventional counterstaining techniques, such as the Papanicolaou stain and the eosin-type stain, produces visible staining patterns familiar to cytologists. The combination has the added advantage of being capable of use with an automated cell analysis system to produce reliable and accurate results which can be used in pathological procedures including, for example, in the detection of cervical cancer.

Brief Description of the Drawing

For a fuller understanding of the nature and objects of the invention, reference should be made to the following detailed description taken in connection with the accompanying drawings, in which:

FIG. 1 is a flow chart depicting the staining technique of the present invention.

FIG. 2 is a graph depicting the absorbance and wavelength of the thionin stain alone and of the thionin stain bound to the nucleus.

FIG. 3 is a flow chart depicting the staining technique of the present invention where a Papanicolaou-type counterstain is used.

FIG. 4 is a flow chart depicting the staining technique of the present invention where an eosin-type counterstain is used.

Detailed Description

A method of staining cellular material according to the invention provides improved contrast of the nucleus from the cytoplasm. The method produces the traditional multicolored cells suitable for manual analysis, and is highly effective in automated analysis systems. The method entails the steps of staining the cellular material with a thionin stain, counterstaining, illuminating the thionin-stained material with energy in the infrared spectrum, and imaging the stained cellular material.

In one practice of the invention, the cells are prepared by the method shown in Fig. 1. The cells are fixed on the slide, step 10, rinsed in two alcohol baths, step 12, and stained with a thionin stain, step 14. The stained cells are rinsed in alcohol baths, step 16, and counterstained, step 18. After counterstaining, the stained cells are rinsed in alcohol, step 20, and rinsed in xylene, step 22, before the coverslip is applied, step 24.

The cells are first fixed on the slide, using alcohol or other methods known in the art, step 10. Then, in preparation for staining, the slide is dipped ten times in each of two baths of high percentage, low molecular weight alcohol, which is preferably methanol, step 12. The cells are stained in a thionin stain solution, step 14, for a time sufficient to incorporate the thionin dye in the nuclei, typically about thirty minutes. After rinsing by dipping ten times in each of three high percentage, low molecular weight alcohol baths, step 16, the cells are counterstained, step 18. That is, the cells are stained with one or more dyes that are primarily taken up by the cytoplasm. When combined with nuclear material, and at the slightly acidic pH, preferably about pH 6.45, thionin dye absorbs infrared light. The counterstain is selected to absorb at a different wavelength from the thionin-stained nuclear material. After staining and counterstaining, the slide is rinsed by dipping in two to four more high percentage, low molecular weight alcohol baths, step 20, and two or more xylene rinses, step 22, before the coverslip is applied, step 24, and the cells are ready to be analyzed, step 26.

When viewed under visible light, the nuclei of the cells are transparent to opaque and stained a deep blue. The cytoplasm is transparent and is multicolored, with the specific color pattern depending on the counterstain used. When cells are stained in this manner, the color pattern is familiar to cytologists, so analysis can readily be carried out by manual, i.e., human, vision. The method has the added advantage in that, when viewed under infrared light, each nucleus is opaque, and the cytoplasm is nearly invisible. With the cytoplasm nearly invisible, overlapping cells will not be confused with nuclei, and an accurate cell count can be easily achieved, manually or by computer.

Solid state television cameras and other opto-electric transducers that are sensitive to infrared energy are employed in the next step, 26, to image the output energy from the stained cells. The image can be digitized for a completely automated analysis and can also be viewed on a display monitor. The nuclear components of the cell are distinguishable and the cells can be measured and classified, e.g., as malignant or as benign.

The thionin stain and method of staining is believed to be primarily responsible for this phenomenon. Thionin alone is not known to absorb infrared light, nor to do so when mixed as a dye or when bound to the nucleus in a Feulgen-type reaction, a highly acidic nuclear stain. However, it has been found that a thionin stain in a buffered solution with a selective acidic range binds only to the nucleus, and becomes strongly absorbing in the near-infrared range. As shown in Fig. 2 with waveform 28, a thionin stain by itself has maximal absorption at 600nm (visible red light), and is almost transparent at around 680nm. When bound to the nucleus, however, the spectrum of absorbance extends to longer wavelengths, as indicated with waveform 30. Peak absorbance remains at about 600nm, but the nuclear-bound thionin continues to absorb into the near-infrared range, and at 780nm, the stained nucleus is absorbing 60 - 70% of its peak.

The thionin stain for practice of the invention can be essentially a thionin salt, an organic solvent, and a pH buffer. In one preferred embodiment, the thionin stain is made of approximately 80 parts of solution A and approximately 20 parts of solution B (1x Buffer) or B_1 (4x Buffer), as prepared according to the following Table I. Solution A is prepared by stirring overnight and then filtering over Whatmann No. 5 filter paper or equivalent.

Table I

Solution A

Thionin acetate (Aldrich)	0.50 gm
Methanol (analytical grade)	100 ml

Solution B

Glacial acetic acid	8 ml
5 N NaOH	18 ml
Distilled water (pH = 4.9)	74 ml

Solution B1

Glacial acetic acid	37 ml
5 N NaOH	63 ml
(pH = 5.24)	

The pH of the thionin solution is preferably approximately 6-7. A thionin stain made of 80 parts solution A and 20 parts solution B or B_1 is slightly acidic at pH = 6.4 +/- 0.1. A stain made with 80 parts solution A and 20 parts solution B_1, has a slightly lower pH at 6.43.

The practice of the invention may be more clearly seen from the following non-limiting examples.

Example I

Papanicolaou-type Stain

One preferred practice of method of the invention combines the thionin stain with the familiar Papanicolaou-type counterstain to attain effective automated analysis. The resultant multicolored stained cells, under visible light, are familiar to cytologists. The traditional Papanicolaou stain is difficult for automated systems to analyze because the variety of colors it produces with the cytoplasm, e.g., blue, green, red, and orange, contrast to varying degrees with the blue stain traditionally used for staining the nucleus. When this blue stain is omitted, in accordance with the invention, and the thionin stain with its unique infrared absorbance is substituted, reliable and accurate automated segmentation, i.e., locating and categorizing the nucleus, is relatively simple. The cytoplasm is analyzed under visible light and segmentation is performed under infrared light, to which solid state television cameras, solid state photo-detectors, and other opto-electric transducing devices are especially sensitive. The resultant image can be digitized, and a cytological analysis can be carried out by computer. Because the stain appears under visible light like the familiar Papanicolaou stain, cytologists can monitor the computerized analysis.

As shown in Fig. 3, the cells are fixed on the slide, step 32, and then the cells are rinsed, step 34, in two separate alcohol baths, prior to staining with a thionin stain, step 36. The stained cells are rinsed in three separate alcohol baths, step 38, stained in the OG6 counterstain, step 40, and rinsed in two alcohol baths, step 42, before staining with an EA solution, step 44. After staining the cells are rinsed in four separate alcohol baths, step 46, followed by three xylene rinses, step 48, and a coverslip or other method of permanent mount, step 50, completes the staining process. After staining, the stained cells are illuminated with infrared light to segment the nucleus, step 52. Imaging step 54 is followed by digitizing step 56 for automated analysis.

In this preferred embodiment, the cells are fixed on the slide using an alcoholic fixing solution or other methods known in the art, step 32. The cells are rinsed by dipping the slide ten times in each of two separate 80% methanol baths, step 34, prior to staining with the thionin stain, prepared according to the above method. After staining for approximately thirty minutes in the thionin bath, step 36, the slides are rinsed by dipping ten times, in each of three separate 95% ethanol baths, step 38, before staining with an OG6 solution, step 40. The OG6 stain is known in the art and is commonly used in Papanicolaou stains. A commercial OG6 stain (Baxter "SP") works well, or it may be preferably prepared by mixing together the ingredients listed in Table II.

Table II

Ingredients	Amounts
Orange G dye	0.5 g
Ethanol	100 ml
Acetic acid	1-5 ml

After staining in the OG6 solution for one minute, the slide is rinsed by dipping ten times in each of two 95% ethanol baths, step 42, before being stained in an EA solution, step 44. This solution, also well known in the art for use in the Papanicolaou staining technique, may be purchased (EA50, Baxter) or prepared by mixing the ingredients listed in Table III.

Table III

Ingredients	Amounts
Eosin Y dye	0.18 g
Light Green dye	0.07 g
Phosphotungsic acid	0.30 g
Ethanol	50 ml
Distilled water	50 ml

After staining in the EA solution for three minutes, the slide is rinsed by dipping ten times in each of four separate 95% ethanol baths, step 46. This is followed by three xylene rinses, step 48, and then followed by coverslipping or other means of permanent mounting known in the art, step 50.

After staining, the cells are illuminated with infrared light to segment the nucleus, step 52. The output energy is imaged for cytological analysis, step 54. The image can be digitized and the analysis is carried out by computer, step 56.

Example II

Thionin-eosin Stain

A second preferred embodiment employs an eosin-type counterstain. When used together, the thionin-eosin stain replaces the standard histological stain, the H-E (hematoxylin-eosin) stain, with one that is easy to analyze with an automated system.

The fixing, rinsing and thionin staining steps are the same as described above and are shown in Figure 4, steps 58-62. After the thionin staining step, the cells are rinsed by dipping ten times in each of three 95% ethanol baths, step 64. The cells are stained in an eosin solution, step 66, prepared by mixing the ingredients found in Table IV. The rinsing, illumination and imaging steps are carried out as described above in Example 1 and shown in Figure 4, steps 68-78.

Table IV

Ingredients	Amounts
Eosin Y dye	1.0 g
Methanol	80.0 ml
Glacial acetic acid	1.6 ml
Distilled water	18.4 ml

It will thus be seen that the objects set forth above, among those made apparent from the preceding description, are efficiently attained and, since certain changes may be made in carrying out the above method without departing from the scope of the invention, it is intended that all matter contained in the above description shall be interpreted as illustrative and not in a limiting sense.

It is also to be understood that the following claims are intended to cover all of the generic and specific features of the invention herein described, and all statements of the scope of the invention which, as a matter of language, might be said to fall therebetween.

Claims

1. A method of staining cellular material for contrasting the nucleus from cytoplasmic material, for producing stained cellular material suitable for automated machine analysis, said method comprising the successive steps of

staining the cellular material with a thionin stain for attaining at least a selected infrared absorbance by nuclear material,

counterstaining the cellular material with a stain having an infrared absorbance by cytoplasmic material distinguishable from said infrared absorbance of nuclear material with said thionin stain,

illuminating the stained cellular material with energy in the infrared spectrum, and

imaging the stained cellular material in response to output energy from the stained cellular material due to said infrared illumination.

2. The method of claim 1 wherein said imaging step comprises digitizing said output energy,

said imaging step further comprising the step of pathologically testing said digitized image information, and

wherein said testing step comprises classifying said cellular material.

3. The method of claim 1 wherein said imaging step includes detecting said output energy with an opto-electric transducer including photomultipliers, solid-state photo-detectors, detector arrays, and television cameras.

4. The method of claim 1 wherein said counterstaining step involves staining with a counterstain compound to produce stained cellular material wherein the cytoplasmic material is relatively transparent to said infrared illumination.

5. The method of claim 1 wherein said staining step includes preparing a thionin stain consisting essentially of a thionin salt, an organic solvent, and a pH buffer.

6. The method of claim 5 wherein said organic solvent is selected from the group consisting of low molecular weight alcohols.

7. The method of claim 6 wherein said low molecular weight alcohol is selected from the group consisting of methanol, ethanol and isopropyl alcohol.

8. The method of claim 1 wherein said infrared illuminating step comprises providing energy in the near infrared spectrum.

9. The method of claim 1 wherein said counterstaining step comprises the steps of

preparing a Papanicolaou-type counterstain consisting essentially of a first staining solution, a second staining solution, and alcoholic rinses,

wherein said first staining solution consists essentially of an orange dye, an alcoholic solvent, and pH buffer, and

wherein said second staining solution consists essentially of a green dye, a red dye, a pH buffer, and an alcoholic solvent,

staining the cellular material, subsequent to said thionin-staining step, with said Papanicolaou-type counterstain,

illuminating the stained cellular material with energy in the infrared spectrum, and

imaging the output radiant energy from said stained cellular material in response to said infrared illumination.

10. The method of claim 1 wherein said counterstaining step comprises the steps of

preparing an eosin-type counterstain consisting essentially of an Eosin Y dye, an organic solvent, and a pH buffer,

staining the cellular material, subsequent to said thionin-staining step, with said eosin-type counterstain,

illuminating the stained cellular material with energy in the infrared spectrum,

imaging the output radiant energy from said stained cellular material in response to said infrared illumination.

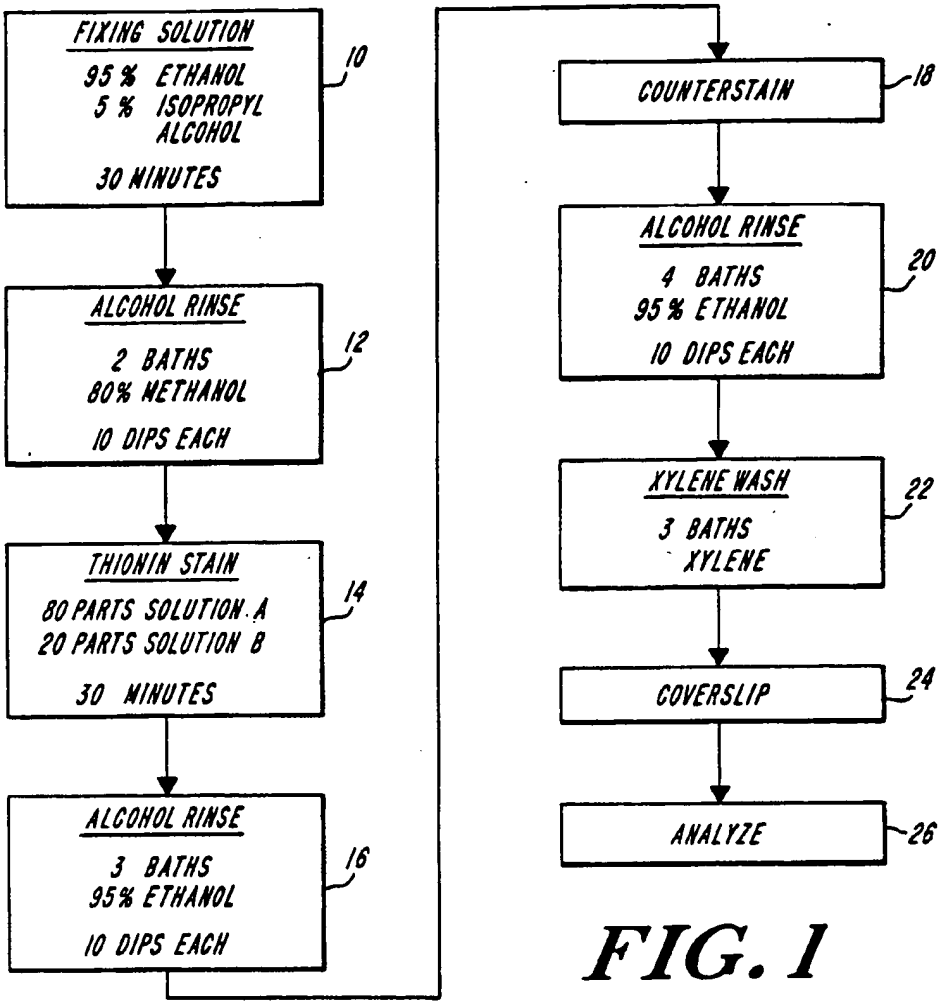

FIG. 1

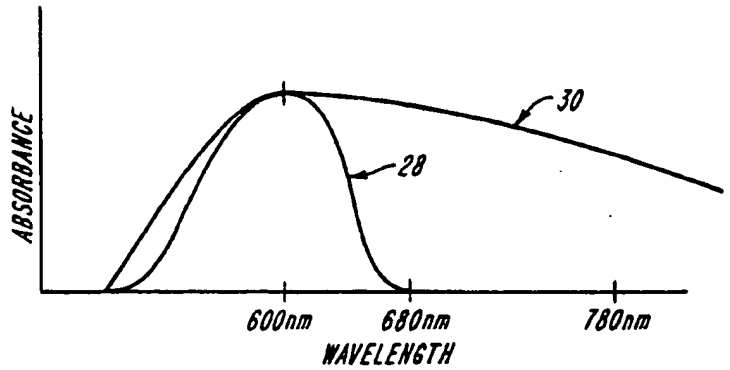

FIG. 2

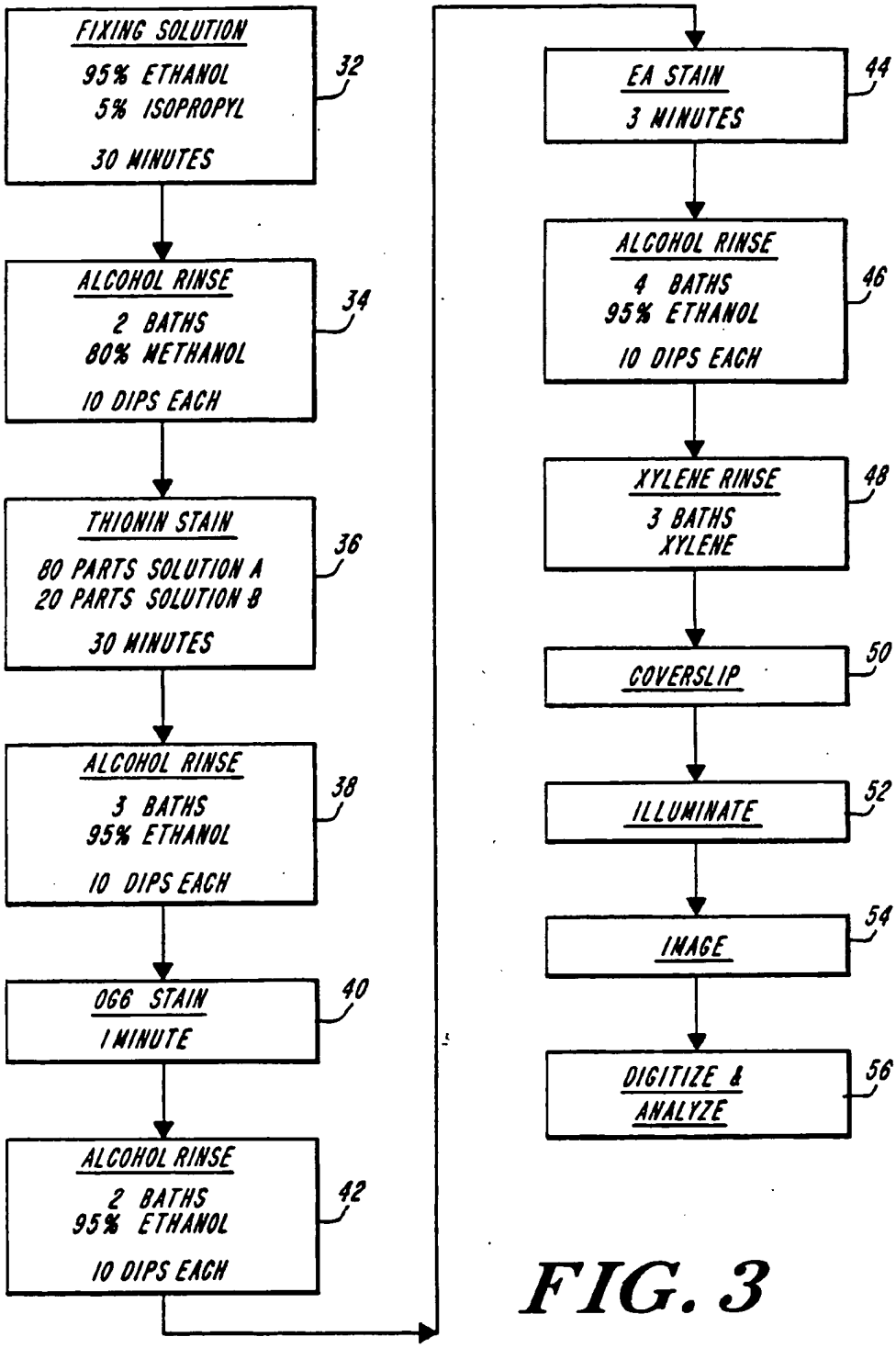
FIXING SOLUTION
95% ETHANOL
5% ISOPROPYL
30 MINUTES
32
ALCOHOL RINSE
2 BATHS
80% METHANOL
10 DIPS EACH
34
THIONIN STAIN
80 PARTS SOLUTION A
20 PARTS SOLUTION B
30 MINUTES
36
ALCOHOL RINSE
3 BATHS
95% ETHANOL
10 DIPS EACH
38
OG6 STAIN
1 MINUTE
40
ALCOHOL RINSE
2 BATHS
95% ETHANOL
10 DIPS EACH
42
EA STAIN
3 MINUTES
44
ALCOHOL RINSE
4 BATHS
95% ETHANOL
10 DIPS EACH
46
XYLENE RINSE
3 BATHS
XYLENE
48
COVERSLIP
50
ILLUMINATE
52
IMAGE
54
DIGITIZE & ANALYZE
56

FIG. 3

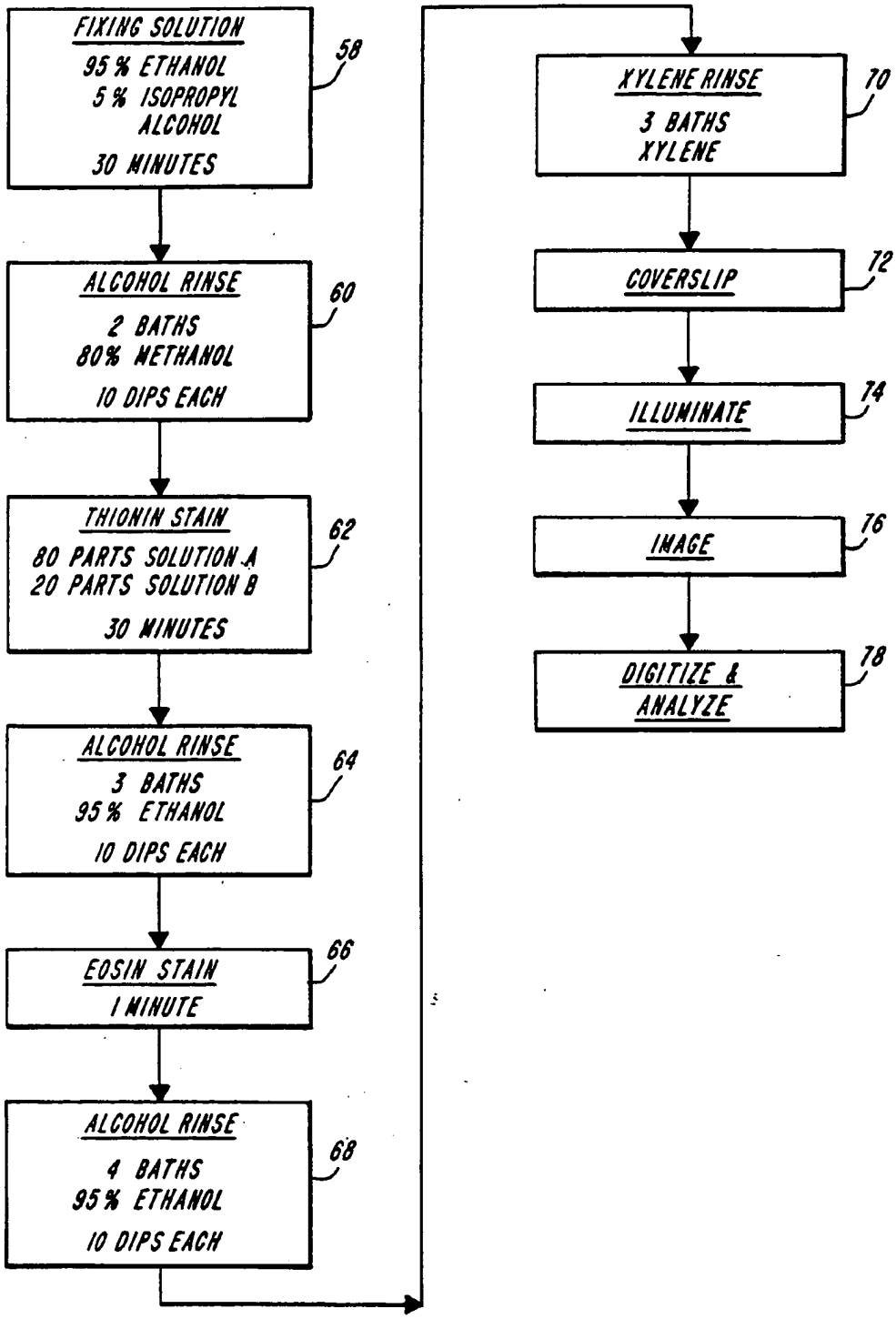
FIXING SOLUTION
95% ETHANOL
5% ISOPROPYL ALCOHOL
30 MINUTES
58
ALCOHOL RINSE
2 BATHS
80% METHANOL
10 DIPS EACH
60
THIONIN STAIN
80 PARTS SOLUTION A
20 PARTS SOLUTION B
30 MINUTES
62
ALCOHOL RINSE
3 BATHS
95% ETHANOL
10 DIPS EACH
64
EOSIN STAIN
1 MINUTE
66
ALCOHOL RINSE
4 BATHS
95% ETHANOL
10 DIPS EACH
68
XYLENE RINSE
3 BATHS
XYLENE
70
COVERSLIP
72
ILLUMINATE
74
IMAGE
76
DIGITIZE & ANALYZE
78

FIG. 4

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Mutational Analysis of a Plant Defensin from Radish (*Raphanus sativus* L.) Reveals Two Adjacent Sites Important for Antifungal Activity*

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Mutational analysis of Rs-AFP2, a radish antifungal peptide belonging to a family of peptides referred to as plant defensins, was performed using polymerase chain reaction-based site-directed mutagenesis and yeast as a system for heterologous expression. The strategy followed to select candidate amino acid residues for substitution was based on sequence comparison of Rs-AFP2 with other plant defensins exhibiting differential antifungal properties. Several mutations giving rise to peptide variants with reduced antifungal activity against *Fusarium culmorum* were identified. In parallel, an attempt was made to construct variants with enhanced antifungal activity by substituting single amino acids by arginine. Two arginine substitution variants were found to be more active than wild-type Rs-AFP2 in media with high ionic strength. Our data suggest that Rs-AFP2 possesses two adjacent sites that appear to be important for antifungal activity, namely the region around the type VI β-turn connecting β-strands 2 and 3, on the one hand, and the region formed by residues on the loop connecting β-strand 1 and the α-helix and contiguous residues on the α-helix and β-strand 3, on the other hand. When added to *F. culmorum* in a high ionic strength medium, Rs-AFP2 stimulated Ca^{2+} uptake by up to 20-fold. An arginine substitution variant with enhanced antifungal activity caused increased Ca^{2+} uptake by up to 50-fold, whereas a variant that was virtually devoid of antifungal activity did not stimulate Ca^{2+} uptake.

During the last decades, it has been recognized that many living organisms produce small antimicrobial peptides to protect their tissues from infectious microbial agents. Well known examples of peptides with antimicrobial properties are the cecropins of invertebrates (reviewed in Ref. 1) and magainins of amphibians (reviewed in Ref. 2). Another class comprises cysteine-rich peptides, among which are the mammalian and insect defensins (3–5), both small, basic proteins with a cysteine-stabilized three-dimensional folding pattern involving antiparallel β-sheets. Insect defensins are produced upon perception of pathogens by the insect fat body and are secreted in the hemolymph (5). Mammalian defensins are present in phagocytic blood cells and are also produced by epithelial cells of the intestines and airways (4). Antimicrobial peptides have also been found in plants. Thionins, for instance, are highly basic 5-kDa peptides toxic to both Gram-positive and Gram-negative bacteria, fungi, yeast, and various mammalian cell types (6). A number of plant species produce thionins constitutively in their seed as well as in their leaves in a pathogen-inducible way (7). Other potent antimicrobial peptides found in plants are structurally related to defensins of mammals and insects and are therefore termed plant defensins (8). Plant defensins are small cysteine-rich peptides consisting of 45–54 amino acids with four intramolecular disulfide bridges. They are encountered in different plant species and various tissues such as seed, flowers, and pathogen-stressed leaves. Comparison of the known primary sequences of a series of plant defensins shows that the arrangement of the cysteines is highly conserved and reveals the existence of a cysteine-stabilized α-helix motif (9), which is also present in insect defensin A (10). Their three-dimensional structure consists of three antiparallel β-strands and an α-helix (11) and is similar to that of insect defensins (5) and some scorpion toxins (*e.g.* charybdotoxin; Ref. 12). Most plant defensins hitherto isolated exhibit antifungal activity. Some of them, for example SIα2[1] (*Sorghum bicolor* inhibitor 2 of α-amylase), are inhibitors of α-amylases but do not inhibit fungal growth (13, 14). The plant defensins with antifungal activity can be divided in two groups. The first group causes morphological distortions of the fungal hyphae resulting in swollen and hyperbranched fungal structures (9, 14). The second group merely inhibits fungal growth without inducing morphological changes. Mode of action studies performed on a representative of each class (Rs-AFP2 from radish and Dm-AMP1 from dahlia) has shown that plant defensins cause rapid ion fluxes upon addition to fungal hyphae, resulting in Ca^{2+} uptake, K^+ efflux, and medium alkalinization (15).

In this study we have performed a structure-function analysis of Rs-AFP2, a plant defensin isolated from radish seed and member of the plant defensin group causing hyperbranching of fungal hyphae. It is the most potent among a number of plant defensin isoforms occurring in radish, including Rs-AFP1 isolated from seed and Rs-AFP3 and Rs-AFP4 isolated from in-

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[1] The abbreviations used are: SIα2, *S. bicolor* inhibitor 2 of α-amylase; AFP, antifungal peptide; AMP, antimicrobial peptide; PCR, polymerase chain reaction; MFα1, mating factor α1; SMF−, synthetic medium fungi; SMF+, synthetic medium fungi with addition of 1 mM $CaCl_2$ and 50 mM KCl; PAGE, polyacrylamide gel electrophoresis.

TABLE I

Oligonucleotides used for PCR-mediated site-directed mutagenesis of Rs-AFP2

Restriction sites are underlined; mutation sites are in bold.

Name	Mutation	Sequence
OWB35		5′-GGAATAGCCGATCGAGATCTAGGAAACAGCTATGACCATG-3′
OWB36		5′-GGAATAGCCGATCGAGATCTAGGA-3′
OWB61		5′-AATAAGCTTGGACAAGAGACAGAAGTTGTGCCAAAGG-3′
OWB41	Q5M	5′-AATAAGCTTGGACAAGAGACAGAAGTTGTGC**ATG**AGGCCAAG-3′
OWB43	T10G	5′-CCAAGTGGGG**G**TTGGTCAGG-3′
OWB45	G16M	5′-GGAGTCTGT**ATG**AACAATAACGC-3′
OWB46	A31W	5′-CTTGAGAAA**TGG**CGTCATGGA-3′
OWB47	F40M	5′-TGCAACTATGTC**ATG**CCAGCTC-3′
OWB48	P41Δ	5′-AACTATGTCTTCGCTCACAAGTG-3′
OWB49	K44Q	5′-TTCCCAGCTCAC**CAA**TGTATCTG-3′
OWB50	Y48I	5′-TGTATCTGC**ATC**TTTCCTTG-3′
OWB77	Y38G	5′-TCTTGCAAC**GGT**GTCTTCCC-3′
OWB162	Y38A	5′-TCTTGCAAC**GCT**GTCTTCCC-3′
OWB161	T10A	5′-CCAAGTGGGG**CTT**GGTCAGG-3′
OWB207	F40A	5′-TGCAACTATGTC**GCT**CCAGCTCAC-3′
OWB51	P7R	5′-TGCCAAAGG**AGA**AGTGGGACATGG-3′
OWB52	G9R	5′-CCAAGT**CGT**ACATGGTCAGG-3′
OWB53	S12R	5′-ACATGG**AGA**GGAGTCTGTGG-3′
OWB54	I26R	5′-AATCAGTGC**AGA**AGACTTGAG-3′
OWB55	L28R	5′-TGCATTAGA**CGT**GAGAAAGCACG-3′
OWB56	N37R	5′-TCTTGC**AGA**TATGTCTTCC-3′
OWB57	V39R	5′-TGCAACTAT**CGT**TTCCCAGCT-3′
OWB58	A42R	5′-GTCTTCCCA**CGT**CACAAGTG-3′
OWB59	I46R	5′-CACAAGTGT**AGA**TGCTACTTTCC-3′
OWB60	F49R	5′-ATCTGCTAC**CGT**CCTTGTTAATAG-3′

fected leaves (8). In order to investigate which amino acids are essential for the antifungal activity of Rs-AFP2, we have undertaken a mutational analysis of this peptide. Information on amino acid substitutions resulting in either a decreased or enhanced antifungal activity, taken together with preliminary data on the three-dimensional configuration of Rs-AFP2, allows prediction of the sites which possibly interact with the yet unknown target site on the fungal hyphae.

MATERIALS AND METHODS

Biological Material—DNA manipulations were performed in *Escherichia coli* strain DH5α. The yeast strain used for expression of the Rs-AFP2 variants was *Saccharomyces cerevisiae* c13-ABYS86 (genotype: *MATα*, *pra1*, *prb1*, *prc1*, *cps1*, *ura3*, *leu2*, *his3*) (16). The fungal strains used for antifungal activity assays were *Alternaria brassicicola* (MUCL 20297), *Ascochyta pisi* (MUCL 20164), *Botrytis cinerea* (JHCC 8973), *Fusarium culmorum* (IMI 180420), *Nectria hematococca* (Collection van Etten 160-2-2), *Phoma betae* (MUCL 9916), and *Verticillium dahliae* (MUCL 19210). Authentic Rs-AFP2 was purified from radish seed as described previously (17).

Site-directed Mutagenesis—Mutagenesis of the Rs-AFP2 coding sequence was performed by two sequential polymerase chain reactions (PCR) as described in Ref. 18. Primers used in the PCR reaction are listed in Table I. In a first PCR, part of the Rs-AFP2 coding sequence was amplified using a sense mismatch primer containing the desired mutation and primer OWB35, a derivative of the M13 reverse primer elongated with a 5′ tag (28 cycles; 1 min at 94 °C, 1 min at 55 °C, 1 min at 72 °C). For design of the mismatch primer, the yeast preferential codon usage was taken into account (19). Ten ng of *Pvu*I-linearized plasmid pBluescript/RsAFP* (20) was used as a template for the first PCR. The amplified product containing the mismatch served as a megaprimer to further elongate the Rs-AFP2 sequence (5 cycles; 1 min at 94 °C, 1 min at 55 °C, 1 min at 72 °C). In a second PCR, this elongated fragment was amplified by primer OWB61, binding to the 5′ end of the Rs-AFP2 gene, and OWB36, an oligonucleotide identical to the 5′ tag of OWB35 (28 cycles; 1 min at 94 °C, 1 min at 55 °C, 1 min at 72 °C). OWB61 contains a restriction site allowing in-frame cloning into the *Hin*dIII site in the MFα1 pro-sequence region of pVD4 (20). Amplification products of the second PCR were digested with *Hin*dIII-*Bam*HI and introduced in the corresponding sites of pVD4. After verification of the occurrence of the desired mutations by nucleotide sequence determination, the expression blocks containing the MFα1 promoter and prepro-sequence followed by the mutated Rs-AFP2 gene were isolated by *Sal*I-*Bam*HI restriction digestion and subcloned into the *Sal*I-*Bgl*II-digested yeast shuttle vector pTG3828 (21). After subcloning, the sequence of the mutated Rs-AFP2 domain was verified by nucleotide sequencing. Restriction enzymes were purchased from Boehringer Mannheim (Mannheim, Germany), T4 DNA ligase from Life Technologies, Inc. (Life Technologies, Merelbeke, Belgium), and *Taq* DNA polymerase from Appligene (Pleasanton, CA). DNA sequencing was performed on a Pharmacia A.L.F. DNA sequencer using the AutoRead Sequencing Kit (Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions.

Heterologous Expression and Purification of Rs-AFP2 Variants—Transformation of *S. cerevisiae*, growth of the yeast cultures, and purification of the Rs-AFP2 variants from the culture supernatants were essentially done as described previously for native Rs-AFP2 (20). Briefly, 250 ml of culture supernatant (minimal selective SD medium: 0.8 g/liter CSM-URA from BIO 101, La Jolla, CA; 6.5 g/liter yeast nitrogen base from Difco; 20 g/liter glucose (Merck); 5 g/liter casamino acids from Difco) was passed over an anion-exchange chromatography column (Q-Sepharose Fast Flow, Pharmacia) connected on-line with a disposable reversed phase C_8 silica column (Bond Elut, 500 mg solid phase, Varian, Harbor City, CA). The C_8 silica column was subsequently rinsed with 6 ml of 10% (v/v) acetonitrile containing 0.1% (v/v) trifluoroacetic acid. The Rs-AFP2 variants were eluted from the latter column with 4 ml of 30% (v/v) acetonitrile containing 0.1% (v/v) trifluoroacetic acid. After drying in a rotating vacuum concentrator, the eluted fractions were purified by reversed-phase chromatography on a C_2/C_{18} silica column (Pep-S, 5-μm beads, 0.4 × 25 cm, Pharmacia). Fractions were collected manually, and the elution position of the Rs-AFP2 variants was determined by a combination of antifungal activity analysis on *F. culmorum* in SMF− (synthetic medium fungi; Ref. 17) and SDS-PAGE analysis. In all cases, elution positions could be determined unambiguously.

Protein Analysis—Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out according to Ref. 22 using a 15% (w/v) acrylamide, 0.5% (w/v) bisacrylamide separating gel and a 5% (w/v) acrylamide, 0.1% (w/v) bisacrylamide stacking gel. Gels were either stained with Coomassie Brilliant Blue R250 or immunoblotted using anti-Rs-AFP1 antibodies as described previously (8). Protein concentrations were determined by the bicinchoninic acid method (23) using authentic Rs-AFP2 as a standard. Free cysteine thiol groups were determined by the Ellman assay on both reduced and unreduced protein samples as described previously (17). Circular dichroism spectra were obtained on a Jasco 600 spectropolarimeter with a cell path of 0.02 cm. Proteins were dissolved at 0.5 mg/ml in distilled water. The spectra were acquired in a single scan mode (10 nm/min) in the ultraviolet region of 265–185 nm. Circular dichroism data were base line-corrected and are presented in units of $\Delta\epsilon$ (M^{-1} cm^{-1}) (24).

Large Scale Purification of Rs-AFP2 Variants—Recombinant yeast (*S. cerevisiae*) cells containing vectors for expression of Rs-AFP2(Y38G) and Rs-AFP2(V39R), respectively, were grown for 7 days in a fermentor

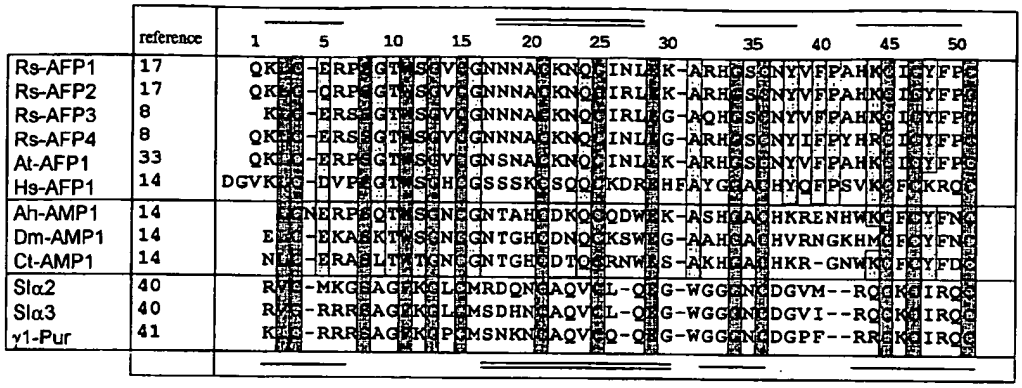

FIG. 1. **Comparison of the complete amino acid sequences of various plant defensins.** Plant defensins of the first subgroup (I) display antifungal activity against *F. culmorum* with concomitant increased hyphal branching. Plant defensins of the second subgroup (II) display antifungal activity without inducing morphological alterations of the fungal hyphae, whereas plant defensins of the third subgroup (III) do not show antifungal activity. Residues conserved among all plant defensins are shown in *dark gray boxes*. Residues conserved among the plant defensins of subgroup I or subgroup I and II but not among plant defensins of subgroup III are presented in *light gray boxes*. The *lines* above the sequence of Rs-AFP1 and underneath the sequence of γ1-Pur indicate secondary structure elements according to Refs. 37 and 11, respectively. *Single lines* represent β-strands and *double lines* α-helices. Abbreviations used are: *Rs-AFP*, *Raphanus sativus* antifungal protein; *At-AFP*, *Arabidopsis thaliana* antifungal protein; *Hs-AFP*, *Heuchera sanguinea* antifungal protein; *Dm-AMP*, *Dahlia merckii* antimicrobial protein; *Ct-AMP*, *Clitoria ternatea* antimicrobial protein; *SIα*, *S. bicolor* inhibitor of α-amylase; *γ1-Pur*, γ1-purothionin.

(Biostat E 15 liter) at 25 °C as a batch-fed culture and harvested at a final OD of approximately 80. Cells were pelleted by centrifugation at 3000 × *g* for 20 min. The supernatants were passed directly over a Biopilot S-Sepharose column (15 × 10 cm, Pharmacia) pre-equilibrated in 20 mM ammonium acetate, pH 6. A flow rate of approximately 100 ml/min was maintained using gravity feed. The bound fraction was eluted with a single wash of 1 liter of 500 mM ammonium acetate (pH 6) and freeze-dried for 3 days to completely remove the ammonium acetate salt. The freeze-dried fraction was dissolved in 20 mM ammonium acetate and refractionated by cation exchange chromatography on a S-Sepharose Fast Flow column (10 × 2.6 cm, Pharmacia) equilibrated in 20 mM ammonium acetate, pH 6. The bound fraction was eluted with a linear gradient of 20–500 mM ammonium acetate (pH 6) over 325 min at 3 ml/min. Proteins were monitored by on-line measurement of absorbance at 280 nm. Fractions containing Rs-AFP2 variants were identified either by using a standard *in vitro* antifungal bioassay (see below) or by Western blot (see above). Fractions containing the expressed peptide were pooled, freeze-dried, and further purified by reversed phase high performance liquid chromatography on a Pep-S column (C_2/C_{18} silica, 25 × 0.93 cm, Pharmacia). Peptides were eluted with a linear gradient of 0.1% (v/v) trifluoroacetic acid to 99.9% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid over 100 min at a flow rate of 3 ml/min. A single predominant peak of absorbance at 280 nm containing the Rs-AFP2 variants was eluted at approximately 20% (v/v) acetonitrile.

Antifungal Activity Assays—The antifungal activity assays were carried out in microplates as described in Ref. 25. A 2-fold dilution series of the protein in sterile water was prepared, and 20 μl of the serial dilutions were added to 80 μl of synthetic low ionic strength medium (SMF−, Ref. 17) containing 10^4 spores/ml of the test fungus without or with the addition of extra salts as indicated under "Results." The plates were incubated at room temperature. Growth of the fungi was monitored microscopically after 24 h and by microspectrophotometry after 72 h unless otherwise indicated. The protein concentration required for 50% growth inhibition (IC_{50} value) was calculated as described in Ref. 17. The specific antifungal activity was defined as $1/IC_{50}$.

$^{45}Ca^{2+}$ Uptake Measurements—*F. culmorum* was grown at an inoculum density of 5 × 10^4 spores/ml in a 100-ml Erlenmeyer flask placed on a rotary shaker (200 rpm). The medium consisted of half-strength potato dextrose broth supplemented with 0.5 μCi of [^{3}H]*N*-acetyl-D-glucosamine/ml (ICN Radiochemicals, Costa Mesa, CA), which merely incorporates into the chitin fraction of the fungal cell wall. The ^{3}H label was used as a measure for biomass, allowing to correct for sample to sample variations in biomass. After 20 h of incubation at 22 °C, 2 μCi/ml $^{45}CaCl_2$ (ICN Radiochemicals) was added, together with the antifungal proteins. After appropriate incubation times, 250-μl samples were taken (in quadruplicate) and transferred to wells of a MultiScreen Durapore 96-well filtration plate (Millipore, Bedford, MA), placed on a MultiScreen vacuum filtration manifold (Millipore). After filtration, harvested hyphae were washed four times with 250 μl of 10 mM $CaCl_2$. Membranes with the hyphae were punched out manually with MultiScreen punch tips (Millipore), and counted for ^{3}H and ^{45}Ca in a liquid scintillation counter (Wallac 1410, Pharmacia).

Preliminary Three-dimensional Solution Structure Determination by ^{1}H NMR—All 51 residues of Rs-AFP1 have been sequence specifically assigned following the strategy of Wüthrich (26) using a combination of double quantum filtered correlation spectroscopy (DQF-COSY) (27), nuclear Overhauser effect spectroscopy (NOESY) (28), and homonuclear Hartmann-Hahn spectroscopy (HOHAHA) (29) spectra recorded on a Bruker AM-500 (Bruker Analytische Messtechnik, Karlsruhe, Germany). These were recorded at a protein concentration of 1.3 mM and pH 4.2, in both 9/1 H_2O/D_2O and D_2O solutions at two different temperatures (304.5 and 313.2 K). The data set at 312.2 K was used to extract 775 nuclear Overhauser effect cross-peaks, and 44 $^3J_{NH\alpha}$ and 62 $^3J_{\alpha\beta}$ coupling constants. Using the programs CALIBA, HABAS, and GLOMSA (30), 775 upper limit constraints, 94 angle constraints, and 19 stereospecific assignments were generated. These data, together with 13 upper and 13 lower limits for the disulfide bridges and the pyroglutamate ring, were used to calculate 500 structures with the program DIANA using the REDAC protocol (30) on a SG Crimson (Crimson, Mountain View, CA). The 25 best structures, with a root mean square deviation of the backbone atoms of all the 51 residues of 1.15 ± 0.22 Å and a root mean square deviation of all the heavy atoms of 1.80 ± 0.24 Å, were optimized by simulated annealing (31, 32) using DISCOVER (AMBER forcefield) and INSIGHT II for visualization (Biosym Technologies, Inc., San Diego, CA). The root mean square deviation of these final structures is 1.33 ± 0.26 Å for the backbone atoms and 1.84 ± 0.28 Å for all the heavy atoms.

RESULTS

Conception and Production of Rs-AFP2 Variants—Proteins belonging to the family of plant defensins have been purified and sequenced from a range of taxonomically divergent plant species, while others have been identified via cDNA sequencing (9). Fig. 1 represents a comparison of the complete amino acid sequences of 12 different plant defensins whose antifungal activity against *F. culmorum* has been assessed in our laboratories (8, 14, 17, 33). In terms of biological activity, three groups of plant defensins can be discerned: group I, those who are inhibitory to *F. culmorum* and cause increased hyphal branching; group II, those inhibitory to *F. culmorum* without causing hyphal deformations; and group III, those not affecting growth of *F. culmorum* at concentrations below 100 μg/ml. Rs-AFP2, the protein studied in this work, belongs to the first group.

As can be seen from the alignment of the sequences in Fig. 1, the pattern of cysteines is totally conserved in all the sequences, as is the glycine residue at position 34 (numbering relative to the studied protein Rs-AFP2). Those residues are important secondary structure elements and are part of the cysteine-stabilized αβ motif characterized by the sequences

TABLE II
Antifungal activity of Rs-AFP2 variants

F. culmorum was grown in a synthetic low ionic strength medium (SMF−) and the same medium supplemented with 1 mM $CaCl_2$ and 50 mM KCl (SMF+). Growth of the fungus was recorded spectrophotometrically after 72 h to determine IC_{50} values. Data are given as mean of triplicates with standard error.

Peptide variant	IC_{50} value on *F. culmorum* in medium	
	SMF−	SMF+
	μg/ml	
Rs-AFP2 (seed)	2.7 ± 0.6	8.5 ± 2.7
Rs-AFP2 (yeast)	2.9 ± 0.8	8.1 ± 2.5
Series 1		
Rs-AFP2(Q5M)	4.1 ± 0.2	5.4 ± 1.2
Rs-AFP2(T10G)	11 ± 4.2	>100
Rs-AFP2(G16M)	2.2 ± 0.3	5.0 ± 0.9
Rs-AFP2(A31W)	30 ± 5.0	>100
Rs-AFP2(Y38G)	42 ± 17	>200
Rs-AFP2(F40M)	16 ± 6.7	54 ± 13
Rs-AFP2(P41Δ)	100 ± 15	>200
Rs-AFP2(K44Q)	3.6 ± 0.4	36 ± 9
Rs-AFP2(Y48I)	9.3 ± 1.0	11 ± 2.0
Series 2		
Rs-AFP2(P7R)	6.8 ± 2.4	8.8 ± 1.0
Rs-AFP2(G9R)	3 ± 0.5	3.3 ± 0.6
Rs-AFP2(S12R)	3.5 ± 1.0	20 ± 6.0
Rs-AFP2(I26R)	7.2 ± 0.8	9.6 ± 3.7
Rs-AFP2(L28R)	6.4 ± 1.4	>100
Rs-AFP2(N37R)	2.8 ± 0.3	7.0 ± 1.8
Rs-AFP2(V39R)	4.0 ± 0.2	3.2 ± 0.3
Rs-AFP2(A42R)	4.2 ± 2.5	18 ± 5.2
Rs-AFP2(I46R)	12 ± 2.4	>40
Rs-AFP2(F49R)	22 ± 4.8	23 ± 3.0

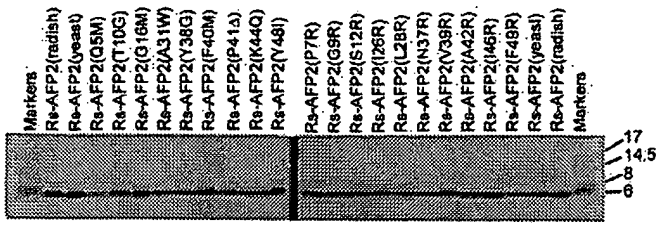

FIG. 2. **SDS-PAGE analysis of Rs-AFP2 variants.** One-μg amounts of Rs-AFP2 variants were separated by SDS-PAGE and stained by Coomassie Brilliant Blue. The molecular masses of the markers are indicated in kDa.

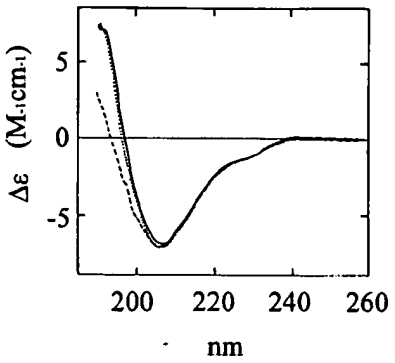

FIG. 3. **Circular dichroism spectra of Rs-AFP2 variants.** Circular dichroism spectra were recorded in a scan from 190 to 260 nm for seed-purified Rs-AFP2 (*solid lines*), Rs-AFP2(Y38G) (*broken lines*), and Rs-AFP2(V39R) (*dots*). The protein concentration was 0.5 mg/ml.

CXXXC, GXC, and CXC (*X* stands for any amino acid) (34). Other well conserved residues are the serine at position 8, the glycine at position 13, and the glutamate at position 29. Those conserved residues were not considered for substitution in the present study, since it is likely that they play a role in determining the structure of the peptide.

A number of amino acid residues were found to be fully conserved among the antifungal plant defensins (group I and II) but subject to non-conservative changes in plant defensins devoid of antifungal activity (group III). Those residues, namely Gln-5, Thr-10, Gly-16, and Ala-31, were considered to be suitable candidate residues for site specific mutational analysis. Lys-44 and Tyr-48, which are conserved in all group I and II plant defensins, except Dm-AMP1 and Hs-AFP1, respectively, were also retained for mutational analysis. In addition, amino acids that are conserved in group I but not in group II could be important for causing the typical morphological deformation of fungal hyphae, which is characteristic for group I plant defensins. These residues comprise Tyr-38, Phe-40, and Pro-41 and were likewise selected for mutational analysis.

A first series of Rs-AFP2 variants was conceived such that the amino acid residues selected as discussed above were substituted by the corresponding residue of SIα2, a group III plant defensin devoid of antifungal activity. Residue Pro-41 was deleted rather than substituted as the loop between the β-strand 2 and β-strand 3 comprising Pro-41 is shorter in group III plant defensins than in group I plant defensins and, furthermore, contains no proline residue in SIα2. It was expected that some of these substitution variants would have a reduced antifungal activity *versus* wild-type Rs-AFP2.

A second series of Rs-AFP2 variants was conceived in such a way that amino acids at selected positions were replaced by the basic amino acid residue arginine. The underlying rationale for these substitutions is that Rs-AFP1, the near-identical but less basic natural analogue of Rs-AFP2, has a lower antifungal activity than Rs-AFP2, especially when assessed in media with a high ionic strength (17). Rs-AFP1 and Rs-AFP2 only differ at two residues (Gln-5 is Glu and Arg-27 is Asn in Rs-AFP1), both of which substitutions result in a higher net positive charge of Rs-AFP2 *versus* Rs-AFP1. This suggests that an increase in the net charge of Rs-AFP2 by replacement of certain residues with arginine might further increase its antifungal activity. The positions selected for the arginine substitutions were those that show weak conservation among the different plant defensins or that are occupied by basic residues in plant defensins other than Rs-AFP2 (see Fig. 1).

For the production of the different Rs-AFP2 variants with the desired amino acid substitution, the Rs-AFP2 coding sequence was mutated site-specifically by PCR, fused in frame to the yeast mating factor α1 (MFα1) promoter and prepro-sequence (20, 21) and subsequently transferred to yeast via a yeast shuttle vector. The different Rs-AFP2 analogues were purified from the yeast culture supernatant by a combination of ion-exchange chromatography and reversed-phase chromatography. Using this approach, we have previously shown that wild-type Rs-AFP2 can be produced in a correctly processed and bioactive form in yeast (20). In total, 19 Rs-AFP2 variants were produced and purified in this way (see Table II). The purity of the preparations was assessed by SDS-PAGE analysis. All Rs-AFP2 variants migrated essentially as single bands which had the same electrophoretic mobility as wild-type Rs-AFP2 (Fig. 2). In addition, all purified proteins were recognized by anti-Rs-AFP1 antiserum on immunoblots prepared from SDS-PAGE gels, confirming their identity as variants of Rs-AFP2 (results not shown).

Two Rs-AFP2 variants, Rs-AFP2(Y38G) and Rs-AFP2(V39R) with a substitution of the tyrosine at position 38 by glycine and of valine at position 39 by arginine, respectively, were purified on a large scale from 15-liter fermentation cultures of the appropriate recombinant yeast strains. Circular dichroism spectroscopic studies were performed on these variants as well as on authentic Rs-AFP2. The circular dichroism spectrum of

Rs-AFP2(V39R) was virtually identical to that of Rs-AFP2, indicating that neither the substitution itself nor the way the variant was synthesized in yeast had imposed alterations of backbone secondary structure elements (Fig. 3). Rs-AFP2-(Y38G) had a circular dichroism spectrum which was almost identical to that of Rs-AFP2, except for a slightly decreased steepness of the $\Delta\epsilon$ drop in the 190–208 nm region (Fig. 3).

Antifungal Activity of Rs-AFP2 Variants—The purified Rs-AFP2 substitution variants were assessed for their antifungal activity against *F. culmorum* in two different media: a low ionic strength medium called SMF– (17), and the same medium supplemented with 1 mM $CaCl_2$ and 50 mM KCl, called SMF+. The presence of salts in the test medium, especially salts with divalent cations, is known to reduce the specific antifungal activity of Rs-AFP2 (17). Seed-purified as well as yeast-expressed wild-type Rs-AFP2 served as controls in the assays. The results of these comparative tests, expressed as IC_{50} values, are presented in Table II. Most of the variants of the first series (substitutions by corresponding SIα2 residues) showed no or only a minor decrease of their antifungal activity in medium SMF–, except Rs-AFP2(A31W), Rs-AFP2(Y38G), and Rs-AFP2(P41Δ), which showed a substantial decrease in antifungal potency. In SMF+, the medium with added salts, a significant decrease in antifungal activity was observed for the following Rs-AFP2 analogues of the first series: Rs-AFP2-(T10G), Rs-AFP2(A31W), Rs-AFP2(Y38G), Rs-AFP2(F40M), Rs-AFP2(P41Δ), and Rs-AFP2(K44Q). On the other hand, the substitutions Q5M and G16M resulted in a slight but significant increase in antifungal potency, especially noticeable in medium SMF+, whereas the substitution Y48I had little or no effect on the antifungal activity.

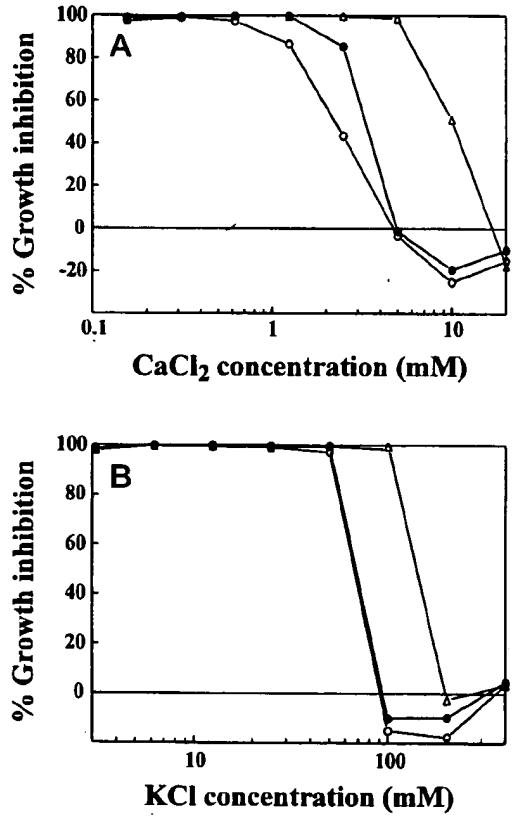

FIG. 4. **Cation sensitivity of the antifungal activity of variant Rs-AFP2(V39R) compared to wild-type Rs-AFP2.** Inhibition of the growth of *F. culmorum* caused by 10 μg/ml of yeast-purified Rs-AFP2 (*open circles*), seed-purified Rs-AFP2 (*closed circles*), and Rs-AFP2(V39R) (*triangles*) in a medium consisting of SMF containing varying concentrations of $CaCl_2$ (*panel A*) and KCl (*panel B*).

In contrast to what was expected, most of the Rs-AFP2 variants of the second series (arginine substitutions) did not show an enhanced antifungal activity compared to Rs-AFP2. In some cases, an even lower antifungal activity was observed, possibly caused by the unfavorable presence of a positive charge at that position or by the absence of a residue necessary for interaction with the fungal target. The largest decrease in antifungal activity was observed for substitution variants Rs-AFP2(L28R) and Rs-AFP2(I46R), whereas variants Rs-AFP2(S12R), Rs-AFP2(I42R), and Rs-AFP2(I49R) showed only a modest reduction in antifungal activity. However, two Rs-AFP2 variants, namely Rs-AFP2(G9R) and Rs-AFP2(V39R), were about 2-fold more active than wild-type Rs-AFP2 when assessed in SMF+.

The antifungal activity of Rs-AFP2(V39R) purified from a large scale culture of recombinant yeast was further characterized in SMF with increasing Ca^{2+} or K^+ concentrations and compared with that of authentic Rs-AFP2 (isolated from seed) as well as yeast-purified Rs-AFP2. As is shown in Fig. 4, the antifungal activity against *F. culmorum* of Rs-AFP2(V39R) was less reduced by the presence of cations in the growth medium in comparison with wild-type Rs-AFP2. Indeed, in the presence of 5 mM $CaCl_2$ and at a concentration of 10 μg/ml, Rs-AFP2(V39R) caused complete inhibition of the growth of *F. culmorum*, whereas wild-type Rs-AFP2 was basically inactive under the same conditions. At 10 μg/ml, wild-type Rs-AFP2 was fully active against *F. culmorum* only when the $CaCl_2$ concentration was equal or lower than 1.25 mM. Likewise, the activity of wild-type Rs-AFP2 was drastically reduced in the presence of 100 mM KCl, whereas Rs-AFP2(V39R) was still fully inhibitory to *F. culmorum* at this KCl concentration.

The potency of Rs-AFP2(V39R) relative to authentic Rs-AFP2 was also assessed on a set of seven different phytopatogenic fungi in three media differing in ionic strength: SMF–, SMF including 1 mM $CaCl_2$ and 50 mM KCl (SMF+), and SMF including 5 mM $CaCl_2$ and 50 mM KCl. As can be seen from the data presented in Table III, the relative antifungal activity of the variant was dependent on the test organism. On three fungi (*F. culmorum, N. hematococca*, and *V. dahliae*), Rs-AFP2(V39R) was more active than Rs-AFP2. In the medium SMF+, for

TABLE III

Antifungal activity of the variant Rs-AFP2(V39R) on different fungal strains and in medium SMF with increasing ionic strength. All IC_{50} values were recorded after 72 h of growth except for V. Dahliae and F. culmorum for which IC_{50} values were determined after 96 h of growth.

Fungus	IC_{50} values (μg/ml)					
	SMF		SMF + 1 mM $CaCl_2$; 50 mM KCl		SMF + 5 mM $CaCl_2$; 50 mM KCl	
	Rs-AFP2	Rs-AFP2(V39R)	Rs-AFP2	Rs-AFP2(V39R)	Rs-AFP2	Rs-AFP2(V39R)
A. brassicicola	3.2	2.5	>50	50	>100	>100
A. pisi	1.9	2.0	>50	>50	>100	>100
B. cinerea	1.8	1.6	>50	>50	>100	>100
F. culmorum	2.1	2.2	4.6	2.3	22	7
N. haematococca	2	2.1	48	9	>100	62
P. betae	0.9	1.4	14	40	27	70
V. dahliae	1	0.4	11	2.3	50	6

instance, Rs-AFP2(V39R) was about 2-, 5-, and 5-fold more potent than Rs-AFP2 against *F. culmorum*, *N. hematococca* and *V. dahliae*, respectively. As in this medium neither Rs-AFP2 nor Rs-AFP2(V39R) inhibited growth of *A. brassicicola*, *A. pisi*, or *B. cinerea* at concentrations below 50 μg/ml, the highest concentration tested, no difference in antifungal potency could be observed for these fungi. However, on *P. betae*, Rs-AFP2(V39R) was less potent than Rs-AFP2. The differences in antifungal potency between Rs-AFP2(V39R) and Rs-AFP2 were always more pronounced in the SMF media with added salts than in the low ionic strength medium SMF−.

Effect of Rs-AFP2 Variants on Ca^{2+} Uptake by Fungi—Although the precise molecular target of Rs-AFP2 on fungal hyphae is not yet known, recent work in our laboratory has shown that Rs-AFP2 causes very rapid ion fluxes, including increased Ca^{2+} uptake, when added to fungal hyphae (15). To investigate whether the ability of Rs-AFP2 to stimulate Ca^{2+} uptake in fungi is linked to its antifungal effect, $^{45}Ca^{2+}$ uptake was measured in *F. culmorum* treated with different concentrations of either Rs-AFP2, the virtually inactive variant Rs-AFP2(Y38G), and the variant with increased antifungal potency, Rs-AFP2(V39R). The medium used for this test consisted of half-strength potato dextrose broth supplemented with 1 mM $MgCl_2$ and 50 mM KCl. As shown in Fig. 5, Rs-AFP2 caused a dose-dependent increase of $^{45}Ca^{2+}$ uptake, which at a dose of 100 μg/ml reached a level that was about 20-fold higher relative to water-treated controls. At the same dose, Rs-AFP2(V39R) stimulated $^{45}Ca^{2+}$ uptake by over 50-fold, and the higher $^{45}Ca^{2+}$ uptake stimulation of Rs-AFP2(V39R) *versus* wild-type Rs-AFP2 was observed over the whole concentration range tested. In marked contrast, however, addition of the variant Rs-AFP2(Y38G) with impaired antifungal properties resulted in $^{45}Ca^{2+}$ uptake rates that fluctuated around the levels observed for water-treated control cultures.

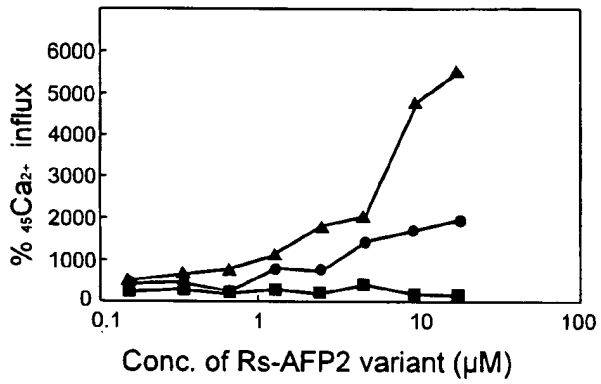

FIG. 5. **Stimulation of $^{45}Ca^{2+}$ uptake in *F. culmorum* by Rs-AFP2 variants.** Yeast-purified Rs-AFP2 (*circles*), Rs-AFP2(Y38G) (*squares*), and Rs-AFP2(V39R) (*triangles*) were added to a suspension of *F. culmorum* (~1 mg biomass dry weight/ml) in half-strength potato dextrose broth supplemented with 1 mM $MgCl_2$ and 50 mM KCl, and $^{45}Ca^{2+}$ uptake was measured 30 min upon addition of the proteins. Values are expressed as relative (in %) to $^{45}Ca^{2+}$ uptake of a suspension treated with water and are means of four replicates.

DISCUSSION

A structure-activity analysis of Rs-AFP2, a plant defensin from radish causing growth inhibition of fungal hyphae (17), was carried out in order to investigate which residues are important for antifungal activity of the peptide. Candidate amino acid residues were considered to be those conserved among plant defensins exhibiting antifungal activity but not among those devoid of antifungal activity as outlined in Fig. 1. Following this rationale, we have chosen to produce a series of nine Rs-AFP2 analogues in which particular amino acid residues were changed to the corresponding residue of the plant defensin SIα2, which does not display antifungal activity. Residue Pro-41 was deleted rather than substituted as the loop encompassing this residue is shorter in SIα2 than in Rs-AFP2. A second series of Rs-AFP2 variants was aimed at increasing the net positive charge (at physiological pH) of Rs-AFP2 by substituting particular residues by an arginine at various non-conserved positions along the Rs-AFP2 sequence. This approach was inspired by the fact that Rs-AFP2, which has a higher net positive charge than Rs-AFP1, has a 2–30-fold higher activity relative to Rs-AFP1 (17).

Wild-type and variant peptides were produced in yeast and purified using identical chromatographic procedures. After the last purification step consisting of reversed phase chromatography, the different peaks were assayed for antifungal activity in order to identify the elution position of the Rs-AFP2 variant. All peptides showed similar retention times. When analyzed by

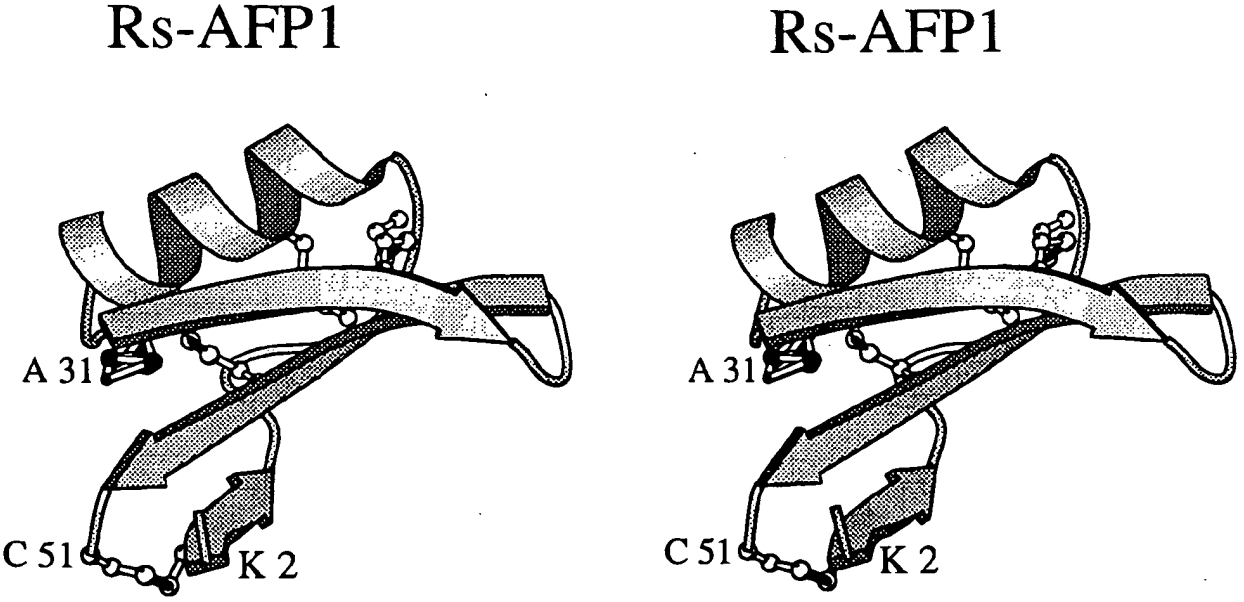

FIG. 6. **Stereoview of Rs-AFP1 with indication of the position of residue Ala-31.** The atoms of residue Ala-31 are shown as *black balls*, while the atoms of the disulfide linkages are shown as *white balls*.

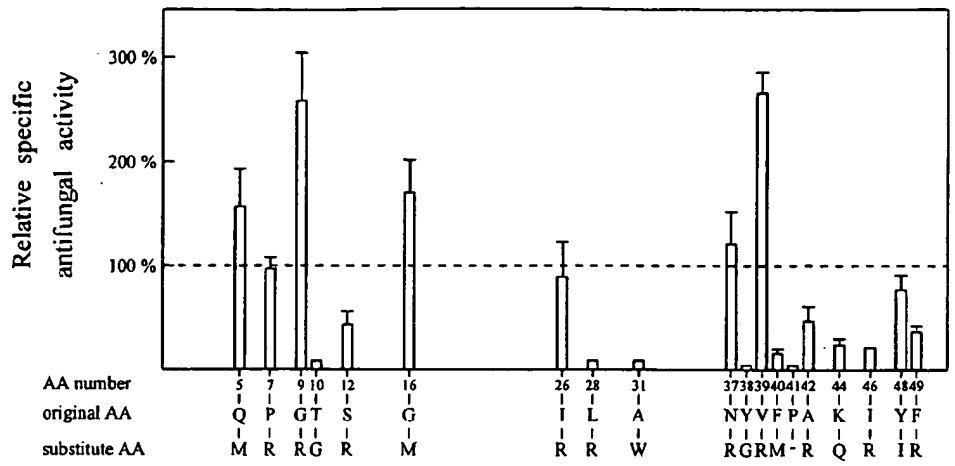

FIG. 7. **Relative specific antifungal activity of the Rs-AFP2 variants as determined against *F. culmorum* in medium *SMF+*.** The specific antifungal activity ($1/IC_{50}$) of Rs-AFP2 was set at 100. *Bars* without indication of standard deviation represent maximum values; actual values may be even lower.

SDS-PAGE, all variant peptides showed the same electrophoretic mobility as wild-type Rs-AFP2, indicating that they have approximately the same size. The structural conformation was studied into more detail by circular dichroism spectroscopy for the variants Rs-AFP2(V39R) and Rs-AFP2(Y38G). In the case of Rs-AFP2(V39R), the circular dichroism spectrum was virtually identical to that of Rs-AFP2, whereas the spectrum of Rs-AFP2(Y38G) showed a slightly altered spectrum in the 190–280 nm region. This alteration is most probably due to the conformational flexibility of glycine within a polypeptide chain. The presence of glycine in the type VI β-turn connecting β-strand 2 and β-strand 3 may entail some relaxation of this region and loosen the packing of the β-sheet. The conformation of the other variants was not verified, but the absence of free thiol groups indicated that the disulfide bridges had formed.

Within the first substitution series, variants that showed a clearly reduced activity on *F. culmorum* were Rs-AFP2(T10G), Rs-AFP2(A31W), Rs-AFP2(Y38G), Rs-AFP2(F40M), and Rs-AFP2(P41Δ). The importance of the residues at positions 10, 38, and 40 is underscored by our observation that substitution variants in which those residues were replaced by an alanine showed a similar drop in antifungal activity.[2] In the second series, consisting of arginine substitution variants, additional variants were identified that displayed reduced antifungal activity, namely Rs-AFP2(S12R), Rs-AFP2(L28R), Rs-AFP2-(A42R), and Rs-AFP2(I46R). It is not clear whether the reduction in antifungal activity of these variants was due to the unfavorable presence of an extra charge or to the replacement of an amino acid essential for the antifungal activity.

Remarkably, the loss in antifungal potency in all these cases was less noticeable in the low ionic strength medium than in the medium supplemented with 1 mM $CaCl_2$ and 50 mM KCl. This may be explained by assuming that the interaction between Rs-AFP2 and its putative receptor on fungal hyphae is based both on ionic interactions and non-ionic stereospecific interactions. Upon increasing the ionic strength of the medium, the ionic interactions with the putative receptor are weakened due to competition between Rs-AFP2 and inorganic cations. In the case where non-ionic stereospecific interactions are weakened due to an unfavorable substitution, the overall interaction is also expected to become more susceptible to ionic competition.

The two most interesting Rs-AFP2 analogues of the arginine substitution series are Rs-AFP2(G9R) and Rs-AFP2(V39R). Although these variants show no significantly increased activity on *F. culmorum* in the low ionic strength medium, their activity on this fungus is much less influenced by the presence of cations in comparison with wild-type Rs-AFP2. This is again consistent with our model, which predicts that the interaction between Rs-AFP2 and its putative receptor is based both on ionic and non-ionic interactions. Introducing an extra charged residue at positions 9 or 39 may reinforce the ionic interactions, leading to variants that are at an advantage in competing with cations for binding at the putative receptor site.

The relative antifungal potency of the arginine substitution variant Rs-AFP2(V39R) compared to Rs-AFP2 appeared to be dependent on the test fungus. Rs-AFP2(V39R) was more active on *F. culmorum*, *N. hematococca*, and *V. dahliae* (three taxonomically related fungi belonging to the family Nectriaceae), but less active on *P. betae*. This suggests that the putative receptor on hyphae of different fungal species may reveal conformational or compositional differences.

As relatively high ionic strength conditions occur in all plant cell compartments (17), Rs-AFP2 variants such as Rs-AFP2(G9R) and Rs-AFP2(V39R) displaying a decreased cation antagonism in their activity against some phytopatogenic fungi could be useful for plant transformation experiments aimed at obtaining disease-resistant crops. We have previously shown that transgenic tobacco plants expressing wild-type Rs-AFP2 are more resistant to the fungal pathogen *Alternaria longipes* than untransformed plants (8). Further enhancement of the resistance level may be achieved through the expression of either Rs-AFP2(G9R) or Rs-AFP2(V39R) in transgenic plants.

We have previously shown that Rs-AFP2 stimulates Ca^{2+} uptake by fungal hyphae, an effect that can be observed within minutes after addition of the peptide (15). This stimulation of Ca^{2+} uptake may be part of the responses triggered by the interaction of Rs-AFP2 with its putative receptor. Our results now seem to indicate that antifungal activity and ability to trigger enhanced Ca^{2+} uptake are correlated. Indeed, the variant Rs-AFP2(Y38G), which is virtually devoid of antifungal activity in presence of inorganic salts, was unable to stimulate Ca^{2+} uptake in *F. culmorum*. On the other hand, the arginine substitution variant Rs-AFP2(V39R) displaying enhanced antifungal potency caused about 2.5-fold higher Ca^{2+} uptake than Rs-AFP2. Controlled Ca^{2+} influx is believed to be essential for directing polar growth at the tip of fungal hyphae (35). For pollen tubes, which like fungal hyphae grow at their tip, it has been documented that various treatments resulting in elevated cytosolic Ca^{2+} levels invariably lead to growth arrest (36).

[2] G. W. De Samblanx, unpublished observations.

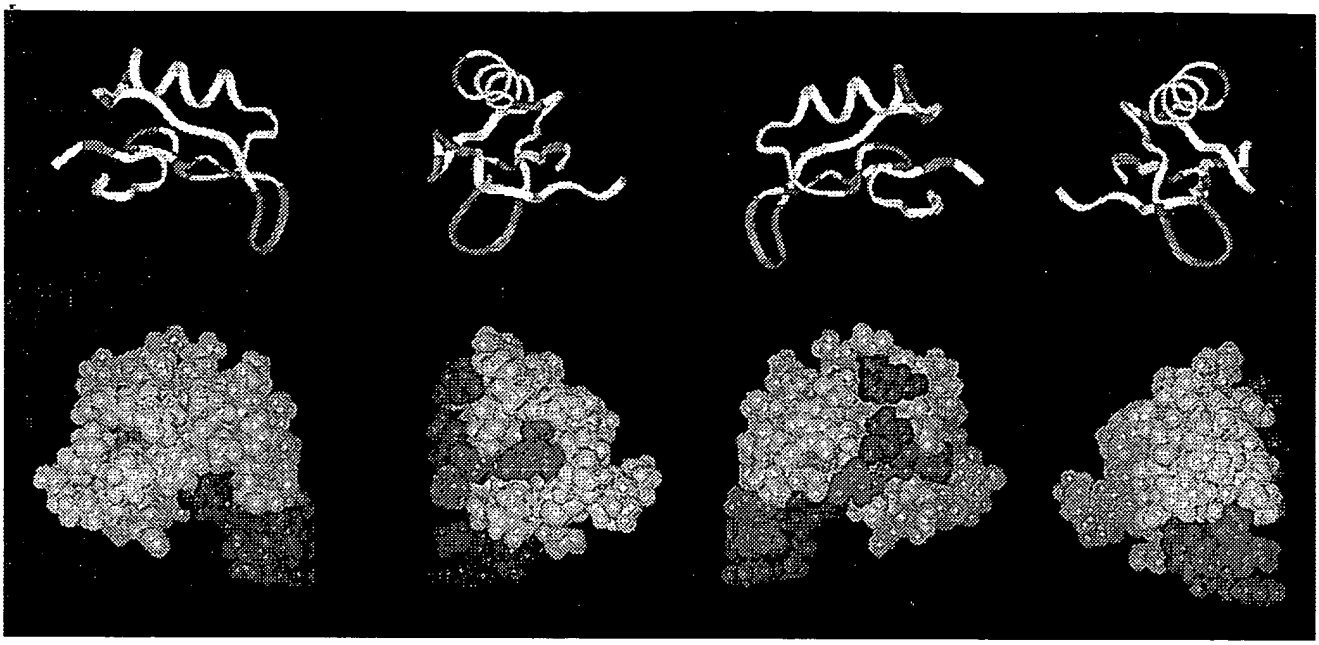

FIG. 8. **Three-dimensional representation of Rs-AFP2 with indication of the residues affecting antifungal activity when substituted.** The Rs-AFP1 molecule is represented in four orientations obtained by rotations of 90° about the vertical axis, with the ribbon presentation of the backbone at the top and the corresponding space-filling models at the bottom. The residues that caused a reduction of the antifungal activity in the medium SMF+ by more than 4-fold when substituted are shown in *dark blue* (Thr-10, Leu-28, Tyr-38, Phe-40, Lys-44, Ile-46), and those which caused a reduction between 2- and 4-fold when substituted are indicated in *light blue* (Ser-12, Ala-42, Phe-49). The residues that enhanced the antifungal activity by more than 2-fold when substituted by arginine are marked in *red* (Gly-9, Val-39). Residue Ala-31, which is likely to entail major conformational changes in the backbone structure when substituted by tryptophan, and the deleted residue Pro-41 are indicated in *green*.

The three-dimensional structure of Rs-AFP1 has been studied by two-dimensional ^{1}H NMR, which has revealed that Rs-AFP1 consists of an α-helix (Asn-18–Leu-28) and a triple-stranded antiparallel β-sheet (β-strand 1: Lys-2–Arg-6; β-strand 2: His-33–Tyr-38; β-strand 3: His-43–Pro-50) (Fig. 6; Ref. 37). Meanwhile, the structure of Rs-AFP1 has been refined down to a root mean square deviation of 1.60 Å for all heavy atoms of the backbone, and the results of this refinement will be presented elsewhere. Since Rs-AFP1 is near-identical to Rs-AFP2, it is assumed that it adopts the same conformation. The spatial orientation of the residues affecting the antifungal activity of Rs-AFP2 upon substitution was analyzed using the high resolution structure of Rs-AFP1. According to the Rs-AFP1 model, all residues substituted in the present study do face outwards of the peptide backbone and are therefore unlikely to be essential for structure stabilization. The only exception is residue Ala-31, which is positioned at the interior face of the hairpin loop connecting the α-helix to β-strand 2 (Fig. 6). Substitution of Ala-31 by a bulky tryptophan residue in Rs-AFP2(A31W) most probably results in a conformational distortion, which might explain the drastic reduction of the antifungal activity of this variant. In addition, deletion of Pro-41, which adopts a cis-configuration in Rs-AFP1 as part of a type VI β-turn, is also likely to entail a distortion of at least the domain encompassing the second and third β-strand and the interconnecting type VI β-turn.

A graphical overview of the specific antifungal activity determined on *F. culmorum* of the different amino acid substitution variants when assayed in high ionic strength medium is provided in Fig. 7. When those residues affecting the antifungal activity are visualized on a three-dimensional model (Fig. 8), it becomes apparent that they all cluster into two adjacent sites. A first site is formed by the residues Tyr-38, Phe-40, Pro-41, Ala-42, Lys-44, and Ile-46. Except for Pro-41 and Lys-44, all those residues are highly hydrophobic. When Lys-44 was substituted by the neutral residue Gln, a substantial decrease of the antifungal potency was observed, suggesting that a positive charge within this predominantly hydrophobic cluster is important for the antifungal activity. This is further substantiated by the observation that the introduction of an additional positive charge within this site at position 39 resulted in enhanced antifungal activity in the presence of inorganic salts. The second site is formed by Thr-10, Ser-12, Leu-28, and Phe-49, which form a patch of contiguous residues despite their scattered positions along the Rs-AFP2 sequence (Fig. 7). Here again, introducing a positive charge within the cluster, namely at position 9, resulted in an enhanced antifungal potency in the high ionic strength medium.

The two regions important for the antifungal activity of Rs-AFP2 might constitute two sites contacting a single putative receptor. Alternatively, the presence of two sites could be indicative of two binding sites on each of two receptor molecules. The latter possibility has been proposed in a model for the interaction between the human growth hormone and its receptor (38). In the case of the human growth hormone, a mutational analysis has also revealed two domains that are involved in the interaction with the human growth hormone receptor. Each of the two domains interacts with a receptor molecule, entailing receptor dimerization, the initial trigger in the signal transduction pathway (39). The physiological meaning of the two functional sites of Rs-AFP2 will remain an open question until its putative receptor has been identified and characterized.

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Fungal Membrane Responses Induced by Plant Defensins and Thionins*

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Treatment of hyphae of *Neurospora crassa* with antifungal plant defensins, *i.e.* Rs-AFP2 and Dm-AMP1 isolated from radish and dahlia seed, respectively, induced a rapid K^+ efflux, Ca^{2+} uptake, and alkalinization of the incubation medium. The Rs-AFP2-induced alkalinization of the incubation medium could be inhibited with G-protein inhibitors. α-Hordothionin, an antifungal thionin from barley seed, caused a sustained increased Ca^{2+} uptake at subinhibitory concentrations but only a transient increased uptake at inhibitory concentrations. α-Hordothionin also caused increased K^+ efflux and alkalinization of the medium, but these fluxes occurred more rapidly compared to those caused by plant defensins. Furthermore, α-hordothionin caused permeabilization of fungal hyphae to the non-metabolite α-aminoisobutyric acid and, in addition, altered the electrical properties of artificial lipid bilayers, consistently leading to rupture of the lipid bilayers. The plant defensins did not form ion-permeable pores in artificial membranes and did not exhibit substantial hyphal membrane permeabilization activity. Our results are consistent with the notion that thionins inhibit fungal growth as a result of direct protein-membrane interactions, whereas plant defensins might act via a different, possibly receptor-mediated, mechanism.

To defend themselves against microbial attack or injury, multicellular organisms produce a battery of antimicrobial peptides and proteins. Many of these peptides and proteins possess broad antimicrobial activity against Gram-positive and/or Gram-negative bacteria, fungi, or enveloped viruses (1). To date, several types of antimicrobial peptides have been isolated, and their structures have been fully or partially characterized. One type of these antimicrobial peptides is small (3–5 kDa), basic, and rich in cysteine. Well studied examples of such cysteine-rich antimicrobial peptides are the insect defensins and mammalian defensins. Insect defensins (34–43 residues; three disulfide bridges) are produced by the insect fat body and secreted in the hemolymph (2). They consist of three distinct domains: an amino-terminal loop; an amphipathic α-helix; and a carboxyl-terminal, double-stranded, antiparallel β-sheet (3). Insect defensins have been shown to disrupt the permeability of the cytoplasmic membrane of *Micrococcus luteus*, resulting from the formation of voltage-dependent ion channels in the cytoplasmic membrane (4). Mammalian defensins (29–34 amino acids; three disulfide bridges) are produced by various specialized cells in the mammalian body (5, 6). Their structure is dominated by three antiparallel β-strands stabilized by three disulfide bridges (7). Mammalian defensins have been shown to form voltage-dependent, weakly anion-selective channels in planar lipid bilayer membranes, and it was suggested that this channel-forming ability contributes to the antimicrobial properties observed *in vivo* (8).

Recently, plant defensins (45–54 amino acids; four disulfide bridges) have been identified as a novel family of cysteine-rich peptides occurring in different plant species (reviewed in Ref. 9). The three-dimensional structure of plant defensins is dominated by a triple-stranded, antiparallel β-sheet and a single α-helix lying in parallel with the β-sheet (10, 11). The α-helix is connected to the second β-sheet via a so-called cystine-stabilized α-helix motif. Plant defensins are structurally related to insect and mammalian defensins, except that insect defensins lack the domain corresponding to the amino-terminal β-strand of plant defensins and that mammalian defensins lack the cystine-stabilized α-helix motif. Several members of the plant defensin family inhibit the growth of a broad range of fungi at micromolar concentrations but are nontoxic to mammalian cells (12–15). At least in radish, it has been shown that an antifungal plant defensin is induced in leaves upon challenge with fungal pathogens, suggesting a role in plant defense (16).

Another group of small cysteine-rich, highly basic peptides, that are thought to play a role in the protection of plants against microbial infection, are the thionins (17). They occur in the seed endosperm, stems, roots, and in etiolated or pathogen-stressed leaves of a number of plant species (18). Their three-dimensional structure can be represented as a compact Γ-shaped molecule. The vertical stem consists of a pair of antiparallel α-helices, and the horizontal arm consists of a short antiparallel β-sheet (19). Thionins are toxic to either Gram-positive or Gram-negative bacteria, fungi, yeasts, and various mammalian cell types. Toxicity requires an electrostatic interaction of thionins with the negatively charged membrane phospholipids, followed by either pore formation or a specific interaction with a certain domain in the membrane (reviewed in Ref. 20). The purely electrostatic interaction of thionins with membranes, the first step in the exposure of toxicity, can be inhibited by divalent cations, *e.g.* calcium (21). The specific interaction of thionins with certain phospholipids, mediating transduction of cellular signals in eukaryotes, can explain the

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release of specific compounds and the activation of calcium channels and specific enzymes upon thionin treatment of mammalian cells (20).

In this study, we have analyzed the membrane responses of *Neurospora crassa*, a saprophytic soil fungus, induced by an antifungal thionin, *i.e.* α-hordothionin (α-HT[1]) from barley seed, and by two antifungal plant defensins, Rs-AFP2 and Dm-AMP1. Rs-AFP2 and Dm-AMP1 belong to two different subgroups of plant defensins, grouped according to the morphogenic effects caused on treated fungal hyphae. Rs-AFP2, an antifungal peptide isolated from the seed of radish (*Raphanus sativus*) (12), belongs to the "morphogenic" plant defensins that cause reduced hyphal elongation with a concomitant increase in hyphal branching. Dm-AMP1, on the other hand, an antifungal peptide isolated from the seed of dahlia (*Dahlia merckii*) (15), belongs to the "nonmorphogenic" plant defensins that slow down hyphal extension but do not induce marked morphological distortions (9, 15).

EXPERIMENTAL PROCEDURES

Materials—Rs-AFP2 and Dm-AMP1 were isolated as described previously by Terras *et al.* (12) and Osborn *et al.* (15), respectively. α-HT was isolated as described by Terras *et al.* (13). $^{45}CaCl_2$, [^{3}H]*N*-Acetyl-D-glucosamine and [^{14}C]α-aminoisobutyric acid were purchased from ICN Radiochemicals (Costa Mesa, CA). Nucleotides [ATPγS, GDPβS and GTPγS] were obtained as lithium salts from Boehringer Mannheim (Laval, Québec, Canada), and carbendazime was from Aldrich (Milwaukee, WI). All chemicals were of reagent grade and were obtained from commercial sources.

Antibiotic Activity Assay—Antifungal activities of protein samples were assayed by microspectrophotometry as described previously (12, 22). Briefly, in a well of a 96-well microplate, 10 μl of the protein sample were mixed with 90 μl of Synthetic Medium Fungi (SMF) (12) containing fungal spores. In the case of testing growth inhibition on pregerminated spores, 90 μl of the appropriate growth medium containing fungal spores were incubated for 20 h. After this period, 10 μl of the protein sample were added. Growth was recorded after 48 h of further incubation at 22 °C. The absorption at 595 nm served as a measure for microbial growth (12). IC_{50}s (*i.e.* the concentration of the antifungal protein that is required to inhibit 50% of the fungal growth) were calculated from dose-response curves with 2-fold dilution steps. The following strains were used: *Neurospora crassa* MUCL 19026 and *Fusarium culmorum* MUCL 30162.

$^{45}Ca^{2+}$ Uptake Measurements—*N. crassa* and *F. culmorum* were grown at inoculum densities of 3×10^5 and 5×10^4 spores/ml, respectively, in a 100-ml Erlenmeyer flask placed on a rotary shaker (200 rpm). The medium consisted of SMF supplemented with 0.5 μCi of [^{3}H]*N*-acetyl-D-glucosamine/ml, which merely incorporates into the chitin fraction of the fungal cell wall. The ^{3}H label was used as a measure for biomass, allowing to correct for sample to sample variations in biomass. After 20 h of incubation at 22 °C, 2 μCi/ml $^{45}CaCl_2$ were added, together with the antifungal proteins. After appropriate incubation times, 250-μl samples were taken (in quadruplicate) and transferred to wells of a MultiScreen Durapore 96-well filtration plate (Millipore, Bedford, MA) placed on a MultiScreen vacuum filtration manifold (Millipore). After filtration, harvested hyphae were washed four times with 250 μl of 10 mM $CaCl_2$. Membranes with the hyphae were punched out manually with MultiScreen punch tips (Millipore) and counted for ^{3}H and ^{45}Ca in a liquid scintillation counter (Wallac 1410; Pharmacia, Uppsala, Sweden). Background counts (membranes without hyphae) were negligible. To relate the ^{3}H counts to biomass, a 10-ml sample of the culture was filtered on a preweighted Millipore glass fiber filter, dried in a vacuum dessicator, weighed, and counted for ^{3}H.

Hyphal Membrane Permeabilization Assay—*N. crassa* or *F. culmorum* were grown at an inoculum density of 3×10^5 and 5×10^4 spores/ml, respectively, in SMF supplemented with 0.5 μCi of [^{3}H]*N*-acetyl-D-glucosamine/ml. After 20 h of incubation, 0.5 μCi of [^{14}C]α-aminoisobutyric acid/ml was added. After an additional incubation of 3 h to allow loading of the hyphae with [^{14}C]α-aminoisobutyric acid, 250-μl samples were taken and transferred to wells of MultiScreen Durapore 96-well filtration plates (Millipore) placed on a MultiScreen vacuum filtration manifold (Millipore). After filtration, hyphae were washed three times with 250 μl of 10 mM α-aminoisobutyric acid. The washed hyphae were then resuspended in 250 μl of SMF containing antifungal proteins. After 3 h of incubation in the presence of the proteins, mycelial suspensions were filtered, and the supernatants were collected into a 96-well microtiter plate and counted for ^{3}H and ^{14}C. The harvested hyphae were washed three times with 250 μl of 10 mM α-aminoisobutyric acid. Membranes with the hyphae were punched out manually with MultiScreen punch tips (Millipore) and counted for ^{3}H and ^{14}C.

K^+ Efflux and External pH Measurements—Twenty-hour-old mycelial suspensions of *N. crassa* and *F. culmorum*, grown in SMF at inoculum densities of 3×10^5 and 5×10^4 spores/ml, respectively, were centrifuged at $3000 \times g$ for 5 min at 20 °C and resuspended after centrifugation in a solution containing 150 μM unbuffered Tris (hydroxymethyl)aminomethane hydrochloride and 10 g/liter glucose. The potassium efflux was monitored by measuring the potassium concentration in the medium with a potassium/valinomycin-selective electrode (Radiometer, Copenhagen, Denmark) as described previously (23, 24), with continuous stirring of the suspension with a magnetic stirring bar. Potassium concentration measurements over time were corrected for electrode drift.

The pH of the suspension was measured using a pH electrode containing protelyte as an electrolyte (Hamilton, Bonaduz, Switzerland), attached to a microprocessor pH meter (pH 537; WTW, Weilheim, Germany) with a chart recorder (S.E. 120; ABB GOERZ, Vienna, Austria).

To determine the stoichiometry between induced H^+ and K^+ fluxes, the potassium efflux and extracellular pH of the mycelial suspensions were simultaneously monitored over a period of 10 min after the addition of the antifungal proteins, as described above. Net H^+ fluxes were determined by titration of the mycelial suspension with 10 mM HCl to the pH of the suspension before addition of the antifungal proteins, which allowed calculation of H^+:K^+ ratios.

Membrane Potential Measurements of N. crassa—Discs (0.2 × 0.2-cm) of mycelium were taken from the periphery of a *N. crassa* culture grown on potato dextrose agar (Difco, Detroit, MI). Each disc was placed mycelial side down on a 1 × 1-cm abraded cellophane square (British Cellophane Ltd.) laid on a Petri dish containing potato dextrose agar. Incubation was at 23 °C overnight.

Intracellular microelectrodes were made from single borosilicate glass capillaries with an inner filament and outside diameter of 1 mm (GC 100F-15; Clark Electromedical Instruments, Reading, United Kingdom). The capillaries were pulled to yield a micropipette with a tip diameter of <0.5 μm (Kopf vertical pipette puller, model 750; Tujunga, CA). Micropipettes were back-filled with 1 M KCl just prior to use. These microelectrodes had resistances of 80–120 MΩ.

The recording chamber consisted of a Petri dish (35 × 10 mm; Corning, New York, NY) in which a hole (18-mm diameter) was milled in the center of the dish. A glass coverslip was pasted over the hole with transparent silicone resin (Sylgard; Dow Corning, Wiesbaden, Germany), creating a recording chamber with a volume of ~1 ml. Just before starting the experiments, a cellophane square containing the mycelium was secured onto the coverslip with high vacuum grease (Corning) and covered with bathing medium (SMF).

The recording chamber was placed on a fixed stage microscope, and cells were viewed with a ×40 long working distance objective. Microelectrode manipulations were performed with a hydraulic micromanipulator (Narishige, Tokyo, Japan). Potentials were measured against an earthed reference chamber, containing a Ag/AgCl pellet and filled with 1 M KCl. All potentials were monitored using a WPI amplifier (World Precision Instruments Inc., New Haven, CT) and a Gould model (DSO) 420 oscilloscope (Gould, Ilford Essex, United Kingdom).

Pore-forming Activity of the Antifungal Proteins—The pore-forming activity of the antifungal proteins was assayed by the planar lipid bilayer technique. Phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, and cholesterol (Sigma) or asolectin (Sigma) and cholesterol were dried under a nitrogen stream and suspended in *n*-decane at a concentration of 20 mg (lipid:cholesterol (80:20))/ml. Müeller-Rudin bilayers were formed from the lipid solutions in *n*-decane in a cell consisting of two compartments connected by an aperture of 0.25-mm diameter. A symmetrical solution containing 10 mM Hepes/KOH (pH 7.2) and 100 mM KCl was used for recordings.

[1] The abbreviations used are: α-HT, α-hordothionin; Rs-AFP2, antifungal peptide isolated from seed of radish (*Raphanus sativus*); Dm-AMP1, antimicrobial peptide isolated from seed of dahlia (*Dahlia merckii*); SMF, synthetic medium fungi; GTPγS, guanosine 5′-*O*-(thiotriphosphate); GDPβS, guanosine 5′-*O*-(thiodiphosphate); ATPγS, adenosine 5′-*O*-(thiotriphosphate).

Different concentrations of antifungal proteins were added to the *cis* chamber (3 ml). All experiments were carried out at room temperature (22 °C). Current was filtered at 1 kHz through a four-pole Bessel filter, recorded on digital audiotapes using a DTR 1200 (Biologic, Claix, France), and digitized using a Digidata 1200 and Axotape software (Axon Instruments, Forster City, CA).

Ion channel activity of the antifungal proteins was also assessed by patch clamp experiments on artificial liposomes obtained by dehydration-rehydration as described (4, 25). Experiments were performed either by perfusing a patch excised from a giant liposome with a solution containing 10 mM Hepes/KOH (pH 7.2), 50 mM KCl, and antifungal proteins or by patch-clamping a liposome in which antifungal proteins had been incorporated previously. Proteoliposomes were formed by incubating small asolectin liposomes, with or without 20% cholesterol, obtained by sonication (1 mg in 300 μl of 10 mM Hepes/KOH, pH 7.2, and 50 mM KCl) for 1 h in the presence of different amounts of antifungal protein. The suspension was then centrifuged at 360,000 × *g* for 30 min at 4 °C, and the pellet was resuspended in 15 μl of 10 mM Hepes/KOH (pH 7.2). Dehydration of the pellet, followed by rehydration in 10 mM Hepes/KOH (pH 7.2) and 50 mM KCl, yielded giant proteoliposomes (5–10 μm) amenable to patch-clamp recording. Two μl of giant liposome suspension were deposited in a Nunclon plastic tissue dish and diluted with 2 ml of bathing solution (10 mM Hepes/KOH (pH 7.2) and 50 mM KCl). Patch electrodes, pulled from Pyrex capillaries (Corning 7740), were filled with a solution containing 10 mM Hepes/KOH (pH 7.2), 50 mM KCl, and 0.4 mM $CaCl_2$ just before use. A Biologic RK 300 amplifier (Biologic, Claix, France) with a 10 GΩ feedback resistor was used. Current was recorded and processed as indicated above for planar bilayer experiments.

RESULTS

Effect of Plant Defensins and Thionins on $^{45}Ca^{2+}$ Uptake in Fungal Hyphae—When administered at 50 μg/ml to hyphae of *N. crassa*, both Rs-AFP2 and Dm-AMP1 caused a drastic increase in the uptake of $^{45}Ca^{2+}$ into the hyphae. The increased $^{45}Ca^{2+}$ uptake was observed within 1 min after addition and reached a plateau after about 30 min at a level which was about 10 times higher than in the untreated hyphae (Fig. 1*A*). In contrast, α-HT added at 50 μg/ml to *N. crassa* caused a very rapid but transient increase of $^{45}Ca^{2+}$, which peaked at 1 min post-application and subsequently dropped to about the level of the control culture after 5 min (Fig. 1*A*). At a subinhibitory concentration of 0.25 μg/ml, however, α-HT induced a rapid and steady elevated $^{45}Ca^{2+}$ uptake level, whereas Rs-AFP2 and Dm-AMP1 did not affect $^{45}Ca^{2+}$ influx at this concentration (Fig. 1*B*).

$^{45}Ca^{2+}$ uptake was also measured 30 min after the addition of the antifungal peptides at different concentrations (Fig. 2). For Rs-AFP2 and Dm-AMP1, the dose-response curves for the increased $^{45}Ca^{2+}$ uptake followed approximately the hyphal growth inhibition dose-response curves. For α-HT, however, maximal $^{45}Ca^{2+}$ uptake was observed at a dose (0.25 μg/ml) that is 40-fold lower than that which yields full inhibition of fungal growth (10 μg/ml).

It has been reported previously that divalent cations inhibit the antifungal activity of Rs-AFP2 (12), Dm-AMP1 (15), and α-HT (26). None of these peptides was found to inhibit the growth of *N. crassa* at a concentration of 50 μg/ml in SMF supplemented with 20 mM $MgCl_2$. When 20 mM $MgCl_2$ was included in the incubation medium, no increased $^{45}Ca^{2+}$ influx could be detected upon addition of 50 μg/ml of the antifungal peptides (results not shown).

Very similar observations on the $^{45}Ca^{2+}$ influx caused by Rs-AFP2, Dm-AMP1, and α-HT were made when the fungus *F. culmorum* was used as a test organism instead of *N. crassa*. In this case, Rs-AFP2 and Dm-AMP1 added at 50 μg/ml caused a 10- and 14-fold higher $^{45}Ca^{2+}$ uptake relative to the control after 30 min, respectively, whereas α-HT caused elevated $^{45}Ca^{2+}$ uptake only at subinhibitory concentrations ranging from 0.5 to 10 μg/ml (results not shown).

Effect of Plant Defensins and Thionins on K^+ Efflux in Fungal Hyphae—At a concentration of 50 μg/ml, α-HT caused *N. crassa* hyphae to very rapidly release K^+ ions into the medium, reaching a half-maximal effect after about 2 min without a detectable lag period. The kinetics of K^+ release induced by α-HT was similar to that of nystatin, a fungicide known to permeabilize fungal membranes (50) (Fig. 3). Both Rs-AFP2 and Dm-AMP1 also caused a substantial efflux of K^+, but the release of K^+ started after a lag period of 0.5–2 min and was somewhat slower compared to that caused by nystatin and thionin (Fig. 3). K^+ efflux could also be detected when the application rates of Rs-AFP2, Dm-AMP1, or α-HT were lowered to 10 μg/ml, but not at 1 μg/ml (results not shown), which is consistent with the doses required for causing hyphal growth inhibition (see Fig. 2).

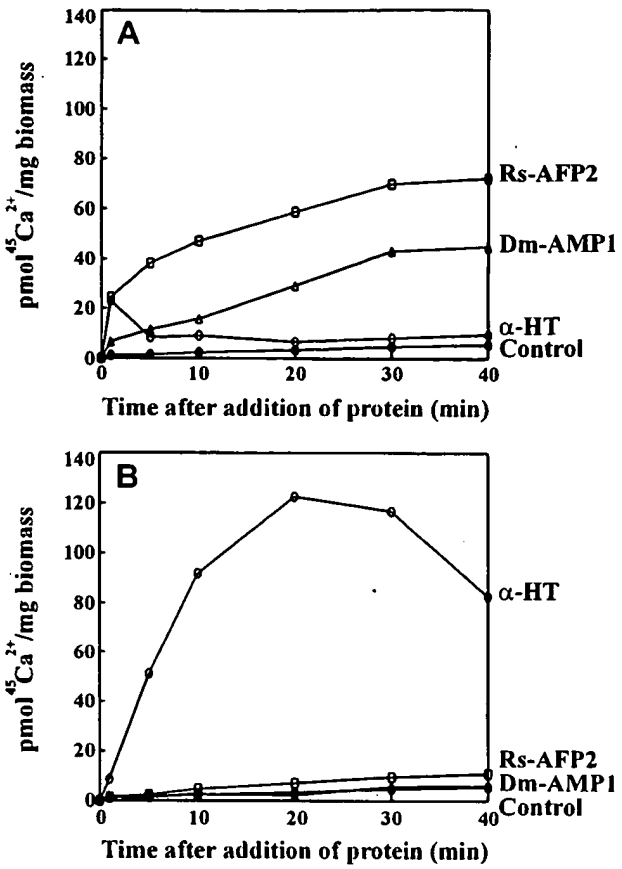

FIG. 1. **The effect of plant defensins and thionin on $^{45}Ca^{2+}$ uptake in *N. crassa* hyphae.** Rs-AFP2 (□), Dm-AMP1 (△), or α-HT (○) was added at either an inhibitory concentration (50 μg/ml; *A*) or a subinhibitory concentration (0.25 μg/ml; *B*) to a suspension of *N. crassa* hyphae (~0.35 mg of biomass/ml) in SMF, and $^{45}Ca^{2+}$ uptake was assayed at different time intervals. ●, $^{45}Ca^{2+}$ uptake by untreated hyphae. Values are expressed as means of four replicates.

Very similar results were obtained when *F. culmorum* hyphae were used instead of *N. crassa* (results not shown). Also in this case, the K^+ efflux caused by α-HT followed similar kinetics as that caused by nystatin and, moreover, was more rapid compared to the K^+ efflux caused by Rs-AFP2 and Dm-AMP1. When either $CaCl_2$ or $MgCl_2$ was included in the incubation medium at 20 mM, no release of K^+ could be detected upon addition of 50 μg/ml of any of the peptides to *N. crassa* hyphae (results not shown).

Effect of Plant Defensins and Thionins on External pH—When applied at 50 μg/ml to *N. crassa* hyphae, Rs-AFP2, Dm-AMP1, and α-HT caused a rapid alkalinization of the medium (Fig. 4). This alkalinization was not observed when the peptides were added to the same medium in the absence of fungal hyphae or when either $CaCl_2$ or $MgCl_2$ were included in the

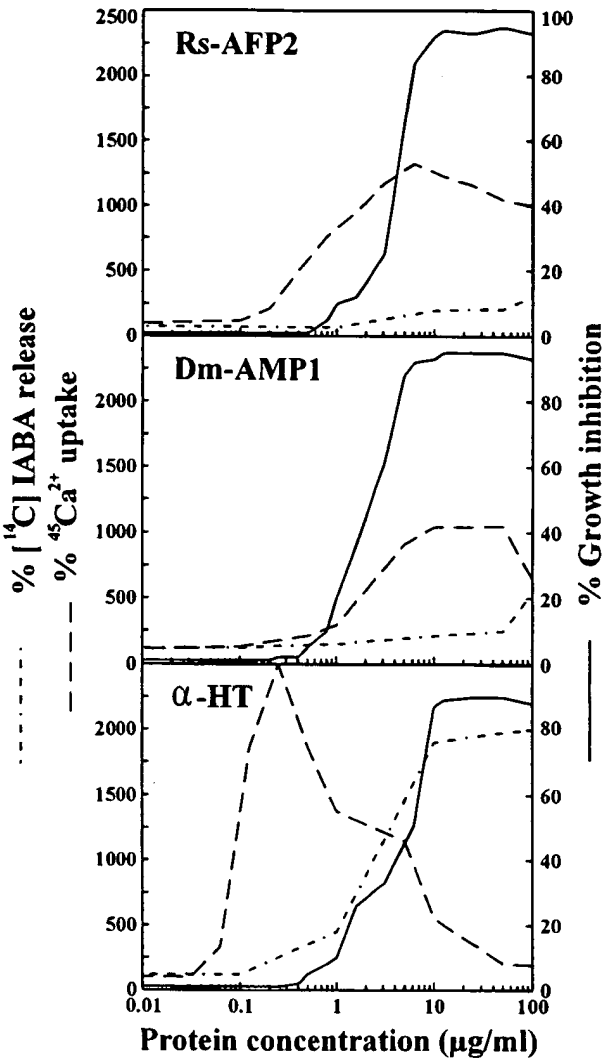

FIG. 2. **The effect of plant defensins and thionin on growth inhibition, $^{45}Ca^{2+}$ uptake, and [^{14}C]α-aminoisobutyric acid release in *N. crassa* hyphae.** Dose-response curves of growth inhibition (———), $^{45}Ca^{2+}$ uptake (— — —), and [^{14}C]α-aminoisobutyric acid (*IABA*) release (— · —) in *N. crassa* hyphae treated with either Rs-AFP2 (*top*), Dm-AMP1 (*center*), or α-HT (*bottom*) are shown. Values are expressed as the means of four replicates.

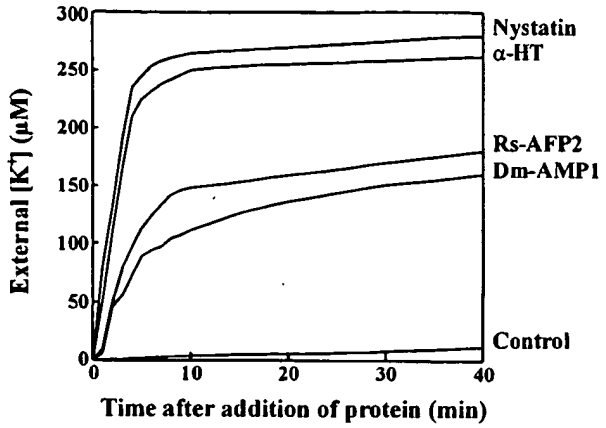

FIG. 3. **Effect of plant defensins and thionin on K^+ efflux in *N. crassa* hyphae.** K^+ efflux into the medium was measured with a K^+/valinomycin selective electrode after the addition of 50 μg/ml of either Rs-AFP2, Dm-AMP1, α-HT, or 100 μg/ml nystatin. The suspension consisted of *N. crassa* (~0.5 mg of biomass/ml) in 150 μM unbuffered Tris and 10 g/liter glucose. Data correspond to one representative experiment of three.

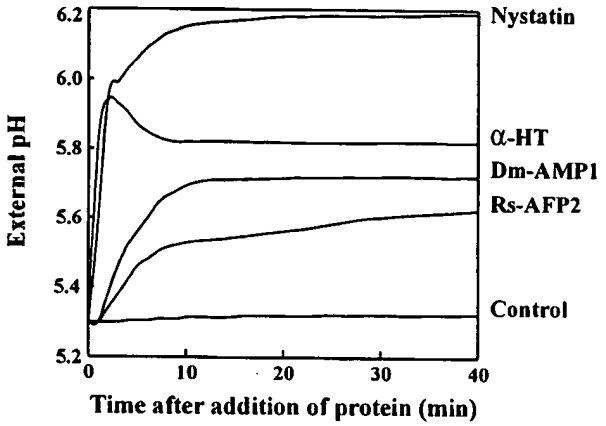

FIG. 4. **Effect of plant defensins and thionin on medium alkalinization in suspensions of *N. crassa* hyphae.** The pH of the medium was measured after the addition of 50 μg/ml of either Rs-AFP2, Dm-AMP1, α-HT, or 100 μg/ml nystatin. The suspension consisted of *N. crassa* hyphae (~0.5 mg of biomass/ml) in 150 μM unbuffered Tris and 10 g/liter glucose. Data correspond to one representative experiment of three.

hyphal suspension at a concentration of 20 mM (results not shown). In the case of α-HT, this alkalinization occurred without a noticeable lag period and reached a half-maximal response after 1 min. Rs-AFP2 and Dm-AMP1 also caused an alkalinization of the external medium, starting after a lag period of 0.5–2 min, and the kinetics of the alkalinization response was slower than in the case of thionin (alkalinization proceeded over a period of 5–10 min after the addition of the proteins). Changes in the pH of the medium were also detected when either Rs-AFP2, Dm-AMP1, or α-HT were added at 10 μg/ml but not at 1 μg/ml (results not shown), which is consistent with the doses required for mycelial growth inhibition (see Fig. 2). Similar alkalinization effects were also observed on *F. culmorum* with all three antifungal peptides. Also in this case, the pH of the medium rose more rapidly when α-HT was applied than when Rs-AFP2 or Dm-AMP1 were added (results not shown).

To investigate a possible coupling between H^+ and K^+ fluxes induced in *N. crassa* by the antifungal peptides, the ratio H^+:K^+ was determined after 10 min of incubation in the presence of the antifungal peptides. The stoichiometry between the H^+ and K^+ fluxes, induced by 20 μg/ml α-HT, was found to be 1.0 ± 0.1 (mean and standard deviation of three experiments). In the case of the plant defensins, a H^+:K^+ ratio of 0.37 ± 0.1 (n = 3) and 0.33 ± 0.12 (n = 3) was measured upon addition of 20 μg/ml of Rs-AFP2 and Dm-AMP1, respectively.

Effect of Guanidine Nucleotide Analogs on Extracellular pH Changes Induced by Plant Defensins and Thionin—As shown above, the addition of plant defensins and thionin to fungal cultures induces a rapid extracellular alkalinization. This readily assayable response was used to assess the responsiveness to the antifungal peptides of fungal cultures pretreated with guanidine nucleotide analogs to test whether G-proteins are involved in the generation of this ion flux. The guanidine nucleotides GTPγS and GDPβS are both inhibitors of G-proteins, locking G-proteins in a GTP-bound active form and GDP-bound inactive form, respectively (27). Both GTPγS and GDPβS reduced the Rs-AFP2 induced extracellular alkalinization by 60% (see Fig. 5) but had only a minor effect on extracellular alkalinization induced by Dm-AMP1 or α-HT. Incubation of fungal cultures with ATPγS, a nucleotide analog which does not affect G-proteins, had little or no effect on extracellular alkalinization induced by plant defensins or thionin.

Effect of Plant Defensins and Thionins on Membrane Poten-

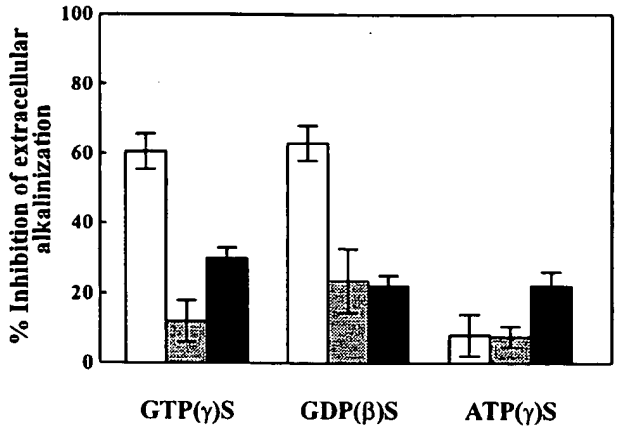

FIG. 5. **Effect of plant defensins and thionin on medium alkalinization in suspensions of *N. crassa* hyphae pretreated with guanidine nucleotide analogs.** The effect of Rs-AFP2 (□), Dm-AMP1 (▨), and α-HT (■) on medium alkalinization in suspensions of *N. crassa* hyphae pretreated with 100 μM of the guanidine nucleotide analogs GTPγS and GDPβS, and ATPγS is shown. The nucleotide analogs were added 15 min prior to the addition of antifungal peptides. The pH of the medium was measured after the addition of 50 μg/ml antifungal peptides. Suspensions consisted of *N. crassa* hyphae (~0.5 mg of biomass/ml) in 150 μM unbuffered Tris and 10 g/liter glucose. Values are expressed as the means of five replicates; *bars*, S.E.

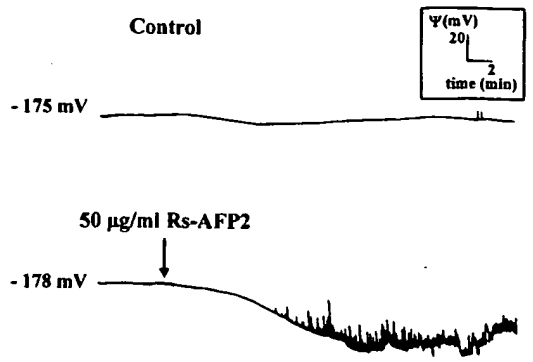

FIG. 6. **Effect of Rs-AFP2 on the membrane potential (ψ) of *N. crassa*.** Membrane potential was recorded while *N. crassa* hyphae were bathing in SMF. *Arrow*, the time at which the addition of 50 μg/ml of Rs-AFP2 occurred. The recording corresponds to one representative experiment of five.

tial of N. crassa—The membrane potential of *N. crassa* hyphae could readily be measured with microelectrodes over at least 60 min. A frequently encountered problem was, however, that the microelectrodes were expulsed from the cytoplasm within 2–5 min after addition of the antifungal peptides, especially Dm-AMP1. This might be due to a gradual loss of cellular turgor, possibly due to K^+ release. Therefore, effects on membrane potential were assessed by impaling microelectrodes in a number of hyphae before and 30–60 min after addition of the proteins. Resting potentials of untreated hyphae were −175 mV ± 4 (n = 12) interior negative. Addition of Rs-AFP2 (20 μg/ml) caused a hyperpolarization of the membrane potential to −210 mV ± 7 (n = 8), whereas Dm-AMP1 (20 μg/ml) caused a moderate depolarization to −144 mV ± 6 (n = 8). Treatment with α-HT (20 μg/ml), on the other hand, resulted in complete depolarization to −36 mV ± 4 (n = 5). Although Rs-AFP2 caused an overall hyperpolarization, membrane potential recordings of Rs-AFP2-treated hyphae showed characteristic trains of transient depolarizing voltage spikes ranging in amplitude from 5 to 50 mV, which appeared after about 5 min (Fig. 6). Such voltage spikes were not observed on recordings of Dm-AMP1 or α-HT-treated hyphae (results not shown).

Effect of Plant Defensins and Thionins on Permeabilization of Hyphal Membranes—To test the effect of the antifungal peptides on permeabilization of the hyphal membranes, *N. crassa* hyphae were incubated in the presence of the nonmetabolizable ^{14}C-labeled amino acid α-aminoisobutyric acid. After uptake of this compound, the hyphae were washed and treated with the peptides, whereafter release of [^{14}C]α-aminoisobutyric acid was measured. As shown in Fig. 2, Rs-AFP2 and Dm-AMP1 only caused a release of the amino acid at the highest concentration tested (100 μg/ml), which is one order of magnitude higher than the minimal concentration required for maximal growth inhibition effect. α-HT, on the contrary, caused a 20-fold increase in isoaminobutyric acid release at 10 μg/ml or higher concentrations, which coincides with the doses required for maximal growth inhibition.

Pore-forming Activity of Plant Defensins and Thionin on Artificial Membranes—The ion channel activity of the antifungal peptides was assessed by the planar lipid bilayer technique. Up to a concentration of 0.6–0.8 μg/ml α-HT in the *cis* compartment, no effect could be observed on an artificial membrane composed of asolectin. Starting from 1 μg/ml or above, α-HT was able to induce a membrane current, indicating permeabilization of the membrane. The typical electrical activity induced by α-HT is displayed in Fig. 7. At 1 μg/ml α-HT, this activity was observed for relatively high values (positive or negative) of the membrane potential (above 80 mV or below −80 mV). Decreasing the membrane potential abolished the activity, and increasing the membrane potential (above +140 mV or below −140 mV) increased the activity and invariably resulted in rupture of the membrane. However, at a higher α-HT concentration (4 μg/ml), this type of electrical activity could be observed even when the membrane potential was kept as low as 30 mV. Under these conditions, rupture of the membrane occurred within 30 s to 2 min after addition of α-HT. At a higher membrane potential (120 mV) and at 4 μg/ml of α-HT, rupture generally occurred in less than 30 s.

In contrast, no such currents were observed when testing either Rs-AFP2 or Dm-AMP1. None of the following attempts to obtain currents by altering the experimental conditions were successful: varying the peptide concentration from 20 to 200 μg/ml; altering the pH from 7.4 to 4.8 in the *cis* chamber; altering the lipid composition to either phosphatidylcholine:phosphatidylethanolamine:phosphatidylinositol:cholesterol (36:32:12:20), which resembles the lipid bilayer composition of *N. crassa* plasma membranes (28), or to phosphatidylcholine:phosphatidylserine:phosphatidylethanolamine:cholesterol (27:27:26:20); and adding either bovine serum albumin (10 mg/ml) or SDS (0.1 mg/ml) to the *cis* chamber (data not shown). Ion channel activity was also assessed by patch clamp experiments on artificial liposomes. Two alternative techniques were explored. (*a*) Giant (proteo)liposomes were prepared in the presence of either R-AFP2 or Dm-AMP1 at concentrations varying from 10 to 100 μg/mg lipid. These proteoliposomes with different lipid composition (asolectin; asolectin + 20% cholesterol; *N. crassa*-like lipid composition (28)) were tested. In none of these proteoliposomes, however, could ion channel activity be observed upon patch clamp recording (n = 27). (*b*) Membrane patches excised from protein-free liposomes with lipid composition resembling *N. crassa* plasma membranes (28) were perfused with a solution containing 20 μg/ml of either Rs-AFP2 or Dm-AMP1, but again no ion channel activity could be recorded (n = 15). It appears, therefore, that Rs-AFP2 and Dm-AMP1 are not able to form ion-permeable pores in the artificial membranes tested.

DISCUSSION

In the present paper, we have shown that two different members of the plant defensin family of antifungal peptides,

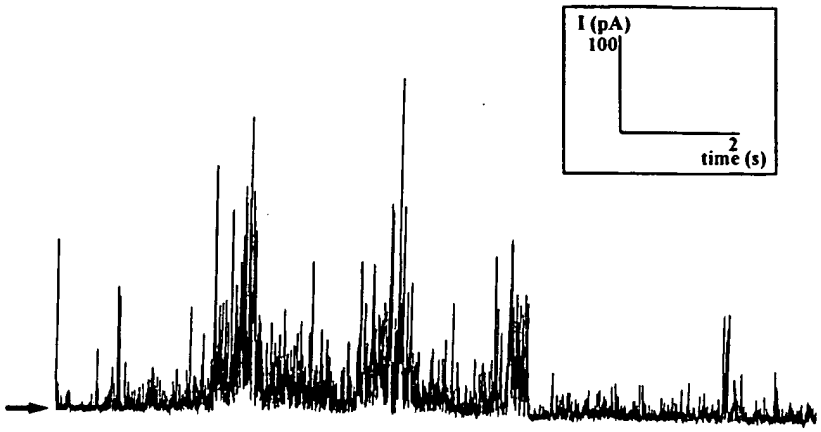

FIG. 7. **Electrical current induced by α-HT in a planar lipid bilayer.** α-HT was added in the *cis* compartment at the final concentration of 2.5 μg/ml. The *cis* and the *trans* compartments both contained 100 mM KCl and 10 mM Hepes/KOH (pH 7.2). The membrane potential was 120 mV. *Arrow*, the zero level current. An increase in membrane current corresponds to an upward deflection.

Rs-AFP2 and Dm-AMP1, induce an array of relatively rapid membrane responses in fungi, including Ca^{2+} uptake, K^+ efflux, alkalinization of the medium, and membrane potential changes. Overall, Rs-AFP2 and Dm-AMP1 elicit very similar responses with similar kinetics, except for their effect on the membrane potential. Indeed, Rs-AFP2 caused the membrane potential of *N. crassa* to hyperpolarize by about 40 mV, whereas Dm-AMP1 caused a partial depolarization by about 35 mV. In fungal hyphae, the membrane potential arises largely from active extrusion of H^+ ions (29, 30). Therefore, the plant defensin-induced modifications of the membrane potential of *N. crassa* can originate from a direct or indirect interaction with the plasma membrane H^+ pump, or alternatively, these induced alterations of the membrane potential can be the result of the triggered ion-fluxes themselves. Since medium alkalinization and Ca^{2+} influx on the one hand, and K^+ efflux on the other hand, have opposite effects on the membrane potential, it is conceivable that the magnitude of the net ion flux induced by either Rs-AFP2 or Dm-AMP1 will cause the membrane potential to either depolarize or hyperpolarize. The membrane potential measurements on hyphae treated with Rs-AFP2 also revealed brief and transient depolarizing pulses that were superimposed on the overall hyperpolarized potential. It is not known at present how these pulses might be generated.

We currently have no direct proof for the linkage between the hyphal growth inhibition effect and the ion fluxes induced by the plant defensins. However, two lines of indirect evidence suggest that both phenomena are inseparable. (*a*) The antifungal activity of both Rs-AFP2 and Dm-AMP1 is inhibited by the divalent cations Ca^{2+} and Mg^{2+} at 20 mM concentrations, which also abolish the increased Ca^{2+} influx, K^+ efflux and external pH changes. (*b*) Similar doses of Rs-AFP2 and Dm-AMP1 are required to induce the ion fluxes as to inhibit fungal growth.

The induced ion fluxes are certainly not the result of growth inhibition since treatment of *N. crassa* hyphae with either 3 mM CN^- (an inhibitor of electron transport chains) or 5 μg/ml carbendazime (an inhibitor of microtubuli formation) (31) did not result in significant variations of $[K^+]$ or $[H^+]$ in the medium or an increased Ca^{2+} influx,[2] although these compounds fully inhibited the growth of *N. crassa* at the concentrations tested (32).

It is believed that a gradient in cytosolic $[Ca^{2+}]$ generated by tip-localized Ca^{2+} channels is essential for driving polarized growth in fungal hyphae (33, 34). This tip-high calcium gradient may orchestrate localized secretion of vesicles containing cell wall building blocks via Ca^{2+}-dependent effector proteins such as annexins (35). In pollen tubes, which like fungal hyphae grow at their tips, it has been shown that treatments resulting in a transient elevation of cytosolic $[Ca^{2+}]$, such as photolysis of caged Ca^{2+} or ionophoretic injection of Ca^{2+}, invariably cause transient growth arrest (36, 37). Moreover, a good correlation was observed between the level of elevated cytosolic $[Ca^{2+}]$ and the extent of growth inhibition (36). It is, therefore, conceivable that the sustained increased Ca^{2+} influx in fungal hyphae treated with plant defensins leads to dissipation of the cytosolic $[Ca^{2+}]$ gradient and thus to hyphal growth inhibition.

How exactly the plasma membrane ion fluxes, including the Ca^{2+} influx, are generated following interaction of fungal hyphae with plant defensins is currently unknown. However, a direct interaction with lipid components of the plasma membrane, as proposed to explain the antimicrobial effects of insect defensins or mammalian defensins (4, 8), is unlikely to be involved for several reasons. (*a*) Proteins that insert in membranes normally display decreased biological activity at lower temperatures. This has been proven to be the case for insect defensins and colicin A (4, 24). We have previously pointed out that the antifungal activity of plant defensins is either temperature insensitive or increases at lower temperature (15). (*b*) Plant defensins did not cause ion channel formation when added to artificial planar lipid bilayers at relatively high concentrations (up to 200 μg/ml). In addition, attempts to record ion channels in artificial liposomes prepared in the presence of plant defensins or perfused with plant defensins were equally unsuccessful. Under identical experimental conditions, an insect defensin from *Phormia terranovae* has previously been shown to form ion-permeable channels in liposomes (4). (*c*) The plant defensins only caused increased hyphal membrane permeabilization, as measured by increased .[^{14}C]α-aminoisobutyric acid release from preloaded hyphae, at a concentration of 100 μg/ml, which is well above the minimal concentration required for growth inhibition. Hence, membrane permeabilization appears to be a secondary effect, which only occurs at high concentrations, but not the primary cause of hyphal growth inhibition. All these data indicate that the ion fluxes result from the interaction of the plant defensins with a receptor that may either transduce a signal to endogenous ion channels in the membrane or, alternatively, facilitate insertion of the plant defensin into the membrane with subsequent ion channel formation. The extracellular alkalinization response elicited by Rs-AFP2 could be inhibited by about 60% by the G-protein inhibitors GTPγS and GDPβS but not by the inactive analog ATPγS. This finding suggests that at least one of the ion fluxes generated by Rs-AFP2 is mediated by a G-protein and, hence, by an endogenous signal transduction component. On the other hand, the guanidine nucleotide analogs had only a

[2] K. Thevissen, unpublished results.

minor effect on the alkalinization response caused by Dm-AMP1 and α-HT.

It is noteworthy that modified ion fluxes have often been reported to occur upon interaction of plant cells or protoplasts with elicitors of defense responses. For instance, when suspension-cultured tobacco cells were treated with α-1,4-D-oligogalacturonides, a rapid stimulation of K^+ efflux was induced, concomitant with membrane depolarization, alkalinization of the incubation medium, acidification of the cytoplasm, and influx of Ca^{2+} (38). The addition of elicitins, a family of small cysteine-rich fungal proteins secreted by *Phytophthora* species, to tobacco cell suspension cultures caused alkalinization of the extracellular medium and concomitant electrolyte leakage (39, 40). Both processes occurred within 2–5 min of elicitin addition. Furthermore, a M_r 42,000 glycoprotein elicitor from *Phytophthora sojae* and a 13-residue elicitor-active peptide derived from the sequence of this glycoprotein were shown to stimulate rapid H^+/Ca^{2+} influxes and K^+/Cl^- effluxes in parsley cells (41). These ion fluxes precede the expression of defense-related genes and phytoalexin biosynthesis (41) and are thought to be mediated by binding of the peptide to a M_r 91,000 plasma membrane protein (42). In cultured tomato cells, the addition of fungus-derived chitin fragments and fungal sterols caused rapid alkalinization of the medium that could be suppressed by the addition of a protein kinase inhibitor K-252a (43, 44). Notwithstanding the fact that a rapid generation of ion fluxes across plant cell membranes has been frequently observed during the interaction of plant cells with fungal compounds, nothing is currently known with certainty about the cellular components in plants which mediate these ion fluxes. It is intriguing, however, that plants secrete peptides such as plant defensins, which appear to impose similar ion fluxes on fungal membranes.

The stoichiometry of K^+ and H^+ fluxes in tobacco cells reacting to either *P. syringae* or oligogalacturonides has been estimated to be approximately 1 H^+:1 K^+ (38, 45). However, much lower and more complex stoichiometries (0.17 H^+:1 K^+) have been described for parsley cells treated with a fungal glycoprotein (46). The stoichiometry of the K^+ and H^+ fluxes induced by Rs-AFP2 and Dm-AMP1 in *N. crassa* was found to be approximately 0.3–0.4. Therefore, it seems unlikely that the induced K^+ and H^+ fluxes are the result of the activation of a simple K^+/H^+ antiport system. Complex kinetics and stoichiometries may result from coupled or uncoupled events mediated by diverse membrane components such as the proton ATPase, H^+/solute co-transporters, and K^+ channels.

In the case of the thionin α-HT, the observed changes in ion fluxes are somewhat different from those induced by plant defensins: $^{45}Ca^{2+}$ uptake is transient at inhibitory concentrations and sustained at subinhibitory concentrations; the membrane potential becomes completely depolarized; K^+ efflux and alkalinization of the medium occur more rapidly and to a higher magnitude when compared to the plant defensins; and the stoichiometry between these induced H^+ and K^+ fluxes is approximately 1 H^+:1 K^+. Furthermore, α-HT was shown to cause increased leakage of α-aminoisobutyric acid and to alter the electrical properties of lipid bilayers. It is, however, doubtful that α-HT forms bona fide ion channels in lipid membranes. Indeed, no clear evidence could be found for the existence of the square-like, more or less correlated fluctuations that are typical of endogenous ion channels as well as of pore-forming toxins. The membrane rupture induced by α-HT was increased at high membrane potential but could, nevertheless, be observed at low membrane potential if the concentration was raised to 4 μg/ml. Wall *et al.* (47) have recently shown that a thionin from *Pyrularia pubera* binds to the surface of artificial membranes but does not insert into them. It is, therefore, probable that thionins exert their permeabilizing activity by binding to membranes and disturbing their organization rather than by forming pores. Membrane permeabilization was also observed in the assay based on α-aminoisobutyric acid release from preloaded *N. crassa* hyphae. The dose-dependence of membrane permeabilization by α-HT correlated well with that of antifungal activity and showed inverse correlation with $^{45}Ca^{2+}$ uptake measured after 30 min. The inverse correlation of $^{45}Ca^{2+}$ uptake and membrane permeabilization was also observed for the plant defensins added at relatively high concentrations (100 μg/ml). It is possible that $^{45}Ca^{2+}$ influx requires cytosolic components that are released upon membrane permeabilization. Alternatively, the decreased $^{45}Ca^{2+}$ uptake might be the result of increased $^{45}Ca^{2+}$ efflux from cytoplasm and vacuoles, due to overall membrane permeabilization. The fact that thionin causes massive uptake of $^{45}Ca^{2+}$ at subinhibitory concentrations implies that increased Ca^{2+} uptake as such is not responsible for growth inhibition in this case. Increased $^{45}Ca^{2+}$ influx and membrane depolarization has previously been found to occur in mouse P388 cells treated with *Pyrularia* thionin (48). It is also well documented that thionins cause leakage of K^+ and other compounds from yeast cells (26) and mammalian cells (49).

All these data are consistent with the notion that thionin affects growth of filamentous fungi mainly by causing membrane permeabilization, most probably due to the binding to the membrane surface and the disturbance of its organization rather than to the formation of pores. Furthermore, the interaction between thionins and fungal cells also leads to secondary effects such as the influx of Ca^{2+}, which may possibly result from the activation of an endogenous Ca^{2+} channel.

Acknowledgments—We thank Dr. A. Taylor (Marine Biological Association, Plymouth, United Kingdom) for assistance in measuring membrane potentials of *N. crassa* and Dr. L. Letellier (Université Paris-Sud, Orsay, France) for helping with K^+ efflux measurements.

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2902171
Derwent Accession: 1987-258442
Utility
EXPIRED
C/ **Method and ophthalmic composition for the prevention and reversal of cataracts**
; **TOPICAL ADMINISTRATION OF THIOREDOXIN**
Inventor: Pigiet, Vincent P., Winchester, MA
Spector, Abraham, New York, NY
Assignee: Trustees of Columbia University in the city of New York(02), New York, NY
COLUMBIA UNIVERSITY (Code: 08871)
Examiner: Brown, J. R. (Art Unit: 183)
Assistant Examiner: Moezie, F. T.
Combined Principal Attorneys: Saliwanchik, Roman; Saliwanchik, David R.; White, John P.

	Publication Number	Kind	Date	Application Number	Filing Date
Main Patent	US 4771036	A	19880913	US 86828112	19860210

Fulltext Word Count: 3845

Summary of the Invention:
...USA, 75, 5827-5830). Other thioredoxin-like peptides include the class of seed proteins called **purothionins** that have intrinsic thioredoxin-like activity (Wada, K. and Buchanan, B. B. [1983] in "Thioredoxins...

Description of the Invention:
...25 mM KCl, 10 mM NaCl, 1.1 mM MgCl[sub]2, 0.1 mM **ethylenediaminetetraacetic** acid (**EDTA**), 10 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES) pH 7.2 (buffer A...

15/3,KWIC/32 (Item 1 from file: 349)
DIALOG(R)File 349:PCT FULLTEXT

00851452
PRODUCTION AND USE OF PROTEIN VARIANTS HAVING MODIFIED IMMUNOGENECITY
VARIANTS DE PROTEINES A IMMUNOGENICITE MODIFIEE
Patent Applicant/Assignee:
NOVOZYMES A S, Krogshojvej 36, DK-2880 Bagsvaerd, DK, DK (Residence), DK (Nationality), (For all designated states except: US)
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VON DER OSTEN Claus, Christian Winthers Vej 15, DK-2800 Lyngby, DK, DK (Residence), DK (Nationality), (Designated only for: US)
Legal Representative:
NOVOZYMES A S (commercial rep.), Krogshojvej 36, DK-2880 Bagsvaerd, DK,
Patent and Priority Information (Country, Number, Date):
Patent: WO 200183559 A2-A3 20011108 (WO 0183559)
Application: WO 2001DK293 20010430 (PCT/WO DK0100293)
Priority Application: DK 2000707 20000428; US 2000203345 20000510; DK

12120265 PMID: 9421187

Mechanisms by which thionin **induces susceptibility of S49 cell membranes to extracellular phospholipase A2.**

Wilson H A; Huang W; Waldrip J B; Judd A M; Vernon L P; Bell J D

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Biochimica et biophysica acta (NETHERLANDS) Nov 15 1997, 1349 (2) p142-56, ISSN 0006-3002 Journal Code: 0217513

Contract/Grant No.: GM-49710; GM; NIGMS

Publishing Model Print

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Languages: ENGLISH

Main Citation Owner: NLM

Record type: MEDLINE; Completed

Subfile: INDEX MEDICUS

Whereas cells normally resist attack by PLA2, they become susceptible under certain pathological conditions. To ascertain the regulatory mechanisms that induce cellular susceptibility to PLA2, the effect of **thionin** on S49 cells was examined in the presence of PLA2. **Thionin** alone was unable to evoke hydrolysis of the lipid bilayer. Likewise, the addition of PLA2 alone caused production of only a minimal amount of free fatty acid. However, **thionin** and PLA2 together resulted in significant hydrolysis of the cell membrane. **Thionin** caused perturbation of the bilayer structure as suggested by the changes in the emission spectra of laurdan and the permeability of the membrane to propidium iodide. These changes correlated quantitatively with the susceptibility of the lipid bilayer to PLA2. Furthermore, **thionin** induced a modest increase in intracellular Ca2+. The source of this Ca2+ was the extracellular fluid since **EDTA** in the extracellular medium inhibited the Ca2+ influx. Moreover, cobalt chloride, a universal Ca2+ channel blocker, prevented the rise in intracellular Ca2+, the uptake of propidium iodide, and the susceptibility to PLA2 induced by **thionin** . In contrast, the changes in the laurdan emission caused by the **thionin** were not affected by the cobalt. Furthermore, incubation of the cells with the calcium ionophore A23187 also caused the cells to become susceptible to PLA2. We hypothesize that **thionin** causes S49 cell membranes to become susceptible to PLA2 by a Ca2+-dependent perturbation of the bilayer structure.

Tags: Research Support, U.S. Gov't, P.H.S.

Descriptors: *Phenothiazines--pharmacology--PD; *Phospholipases A --pharmacology--PD; Animals; Arachidonic Acid--metabolism--ME; Calcimycin --pharmacology--PD; Calcium--metabolism--ME; Cell Membrane--metabolism--ME; Lipid Bilayers--metabolism--ME; Lymphoma--metabolism--ME; Mice; Tumor Cells, Cultured

CAS Registry No.: 0 (Lipid Bilayers); 0 (Phenothiazines); 506-32-1 (Arachidonic Acid); 52665-69-7 (Calcimycin); 581-64-6 (thionine); 7440-70-2 (Calcium)

Enzyme No.: EC 3.1.1.- (Phospholipases A)

Record Date Created: 19980115

Record Date Completed: 19980115

5/9/2

DIALOG(R)File 155:MEDLINE(R)

10106631 PMID: 7680580

Thionin **staining of paraffin and plastic embedded sections of cartilage.**

Bulstra S K; Drukker J; Kuijer R; Buurman W A; van der Linden A J

Department of Orthopaedic Surgery, University Hospital Maastricht, State University of Limburg, The Netherlands.

Biotechnic & histochemistry - official publication of the Biological Stain Commission (UNITED STATES) Jan 1993, 68 (1) p20-8, ISSN 1052-0295 Journal Code: 9107378

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Document type: Journal Article

Languages: ENGLISH

Main Citation Owner: NLM

Antifungal activity of the small and large subunit of 2S albumins.

The small...

...antifungaleffect on F culmorum was assessed for combination between oxidised SS or LS and a- **purothionin** by using the same approach as described in Example 7. The results of these experiments...

...SYNERGISTIC ANTIFUNGAL EFFECT ON FUSARIUN CULNORUN OF CONBINATIONS BI OXIDISED SS OR LS AND w- **PUROTHIONIN**
Test protein IC 50 of a- **purothionin** , og/ml (Synergism Factor)
Nedium A Medium B
Test protein conc. Test protein conc.
(Pg...

...LS (though to a much lesser extent) are able to potentiate the activity of a.- **purothionin** , A synergism factor of up to 33 could be obtained when oxidised SS was added at 10 pg/ml to a lution of a.- **purothionin** and assayed in medium A. In medium B a synergism factor of 2 was measured...14576-14581) and also exert antifungal activity and display the synergistic effect with the a- **purothionin** (see Examples 5 and 10), this substitution is not believed to affect nor the antifungal...

15/3,KWIC/60 (Item 29 from file: 349)
DIALOG(R)File 349:PCT FULLTEXT

00224457
BIOCIDAL PROTEINS
PROTEINES BIOCIDES
Patent Applicant/Assignee:
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BROEKAERT Willem Frans,
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Inventor(s):
BROEKAERT Willem Frans,
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REES Sarah Bronwen,
VANDERLEYDEN Jozef,
Patent and Priority Information (Country, Number, Date):
Patent: WO 9221699 A1 19921210
Application: WO 92GB999 19920603 (PCT/WO GB9200999)
Priority Application: GB 9112300 19910607
Designated States:
(Protection type is "patent" unless otherwise stated - for applications prior to 2004)
AT AU BB BE BF BG BJ BR CA CF CG CH CI CM CS DE DK ES FI FR GA GB GN GR HU IT JP KP KR LK LU MC MG ML MN MR MW NL NO PL RO RU SD SE SN TD TG US
Publication Language: English
Fulltext Word Count: 7952

Fulltext Availability:
Detailed Description

Detailed Description
... buffer containing 10 MM NaH 2POV 15 mm Na2HPO 41 100 MM KC1, 2 MM **EDTA** , 2 MM thiourea, 1 MM

PMSF and 1 mg/l leupeptin. The homogenate was squeezed...buffer contained 200'mM TriS-HC1 (pH 8*3), 1% (w/v) SDSI I mM **EDTA** , 0,005% bromophenol blue and, unless otherwise stated, 1% (w/v) dithiothreitol (DTT), Proteins were...

...6 M guanidinium-Cl containing 100 mM sodium phosphate buffer (pH 7) and I mM **EDTA** , The mixtures were allowed to react with 5,51-dithionitrobenzoic acid and monitored for release...AMP1 nor Ac-AMP2 affected cell viability after 24 h of incubation. In contrast, A- **purothionin** administered at 50 pg/ml decreased the viability of both cell types by more than...

15/3,KWIC/61 (Item 30 from file: 349)

00218464

BIOCIDAL PROTEINS

PROTEINES BIOCIDES

Patent Applicant/Assignee:
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DE BOLLE Miguel,
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Inventor(s):
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BROEKAERT Willem Frans,
CAMMUE Bruno Philippe Angelo,
VANDERLEYDEN Jozef,
REES Sarah Bronwen,

Patent and Priority Information (Country, Number, Date):
Patent: WO 9215691 A1 19920917
Application: WO 92GB423 19920310 (PCT/WO GB9200423)
Priority Application: GB 915052 19910311; GB 915684 19910319
Designated States:
(Protection type is "patent" unless otherwise stated - for applications prior to 2004)
AT AT AU BB BE BF BG BJ BR CA CF CG CH CH CI CM CS DE DE DK DK ES ES FI FR GA GB GB GN GR HU IT JP KP KR LK LU LU MC MG ML MN MR MW NL NL NO PL RO RU SD SE SE SN TD TG US
Publication Language: English
Fulltext Word Count: 6953

Fulltext Availability:
Detailed Description

Detailed Description
... extraction buffer containing 10 mM NaH2POV 15 mM Na 2HP041 100 mM KC1. 2 mM **EDTA** , 2 mM thiourea, I mM PMSF and 1 mg11 leupeptin. The homogenate was squeezed through...buffer contained 200 mM Tris-HCI (pH 8.3), 1% (w/v) SDS, I mM **EDTA** , 0.005% bromophenol blue and, unless otherwise stated, 1% (w/v) dithiothreitol (DTT). Silver staining...dioica agglutinin or UDA (Broekaert, WF et al; 1989; Science, 245, 1100-1102) and 0- **purothionin** (Hernandez-Lucas, C et al; 1974; Appl Microbiol, 28, 165-168). Fungi were grown on...
...as previously described (Peumans, Wi et al; 1983; FEBS Lett, 177, 99-103). The 0- **purothionin** was purified from wheat

endosperm by the method of Redman, DG and Fisher, N (1969...

...Table 2 summarises the results. Serial dilutions of Mj-AMP1, Mj-AMP21 UDA and 0- **purothionin** were applied to fungi and the percent growth inhibition measured by microspectrophotometry (as described in...20 jig/ml for Mj-AMP2. from 0.5 to 15 jig/ml for a- **purothionin** , and from 20 to over 1,000 /ig/ml for UDA depending on the test...

...on an average basis the obtained antifungal activity series is as follows: Mj-AMP2 = 0- **purothionin** > Mj-AMP1 > UDA, Some fungi, such as B cinerea, C lindemuthianum and V inaegualis, are clearly more sensitive to Mj-AMP2 than to 0- **purothionin** . Conversely, the latter protein is most effective in deterring growth of other fungi such as...

...time-dependent drop in antifungal activity, however, was less pronounced for Mj-AMP2 and P- **purothionin** than for Mj-AMP1 or UDA.

ALsor Mj-AMP2 and 0- **purothionin** characteristically produced steeper dose-response curves than Mi-AMP1 or UDA. Figure 5 shows the...

...and B), Mj-AMP2 (panels C and D), UDA (panels E and F), and a- **purothionin** (panels G and H). The percent growth inhibition was recorded after 48 h (0 ---- 0...positive and gram-negative bacteria: Bacillus megaterium, Sarcina lutea, Escherichia coli and Erwinia carotovorao A- **purothionin** and UDA were also tested for comparison (see Example 7). Tests were performed in soft...

...hours, Results are shown in Table 6.

TABLE 6
Antibacterial activity of Mj-AMPs, 9- **purothionin** and UDA
Bacterium IC 50 (/Jg/ml)
Mj-AMP1 Mj-AMP2 13-pt UDA
MEDIUM...

15/3,KWIC/62 (Item 31 from file: 349)
DIALOG(R)File 349:PCT FULLTEXT

00208988
PROTEINACEOUS ANTI-DENTAL PLAQUE AGENTS
AGENTS D'ELIMINATION DE LA PLAQUE DENTAIRE A BASE DE PROTEINES
Patent Applicant/Assignee:
PROTEIN ENGINEERING CORPORATION,
LADNER Robert Charles,
GUTERMAN Sonia Kosow,
Inventor(s):
LADNER Robert Charles,
GUTERMAN Sonia Kosow,
Patent and Priority Information (Country, Number, Date):
Patent: WO 9206191 A1 19920416
Application: WO 91US7099 19910927 (PCT/WO US9107099)
Priority Application: US 90657 19900928; US 91989 19910301
Designated States:
(Protection type is "patent" unless otherwise stated - for applications prior to 2004)
AT AT AU BB BE BF BG BJ BR CA CF CG CH CH CI CM CS DE DE DK DK ES ES FI
FR GA GB GB GN GR HU IT JP KP KR LK LU LU MC MG ML MN MR MW NL NL NO PL

RO SD SE SE SN SU TD TG US
Publication Language: English
Fulltext Word Count: 13547

Fulltext Availability:
Detailed Description

Detailed Description
... human inter-alpha
trypsin inhibitor (58AAF 3 -SS-), crambin (46 AA; 3 -SS-)f
alpha **purothionin** (45 AA; 4 -SS-), beta **purothionin** (same),
human secretory leukocyte protease inhibitor (107 AA; 8 -SS
30), hen egg-white lysozyme...of 0,1 to 12% by weight.

The composition may comprise a humectant, such as
polyethylene glycol, **ethylene** glycol, sorbitol, glycerol,,
propylene glycol, 1,3-butylene glycol, xylitol, maltilol,
lactitol, and the like...tablet may optionally be coated with a
coating material such as waxes, shellac, carboxymethyl
cellulose, **polyethylene** /maleic anhydride co-polymer or
Kappacarrageenan to further increase the time it takes the
tablet...

15/3,KWIC/63 (Item 32 from file: 349)
DIALOG(R)File 349:PCT FULLTEXT

00159749
USE OF THIOREDOXIN, THIOREDOXIN-DERIVED, OR THIOREDOXIN-LIKE DITHIOL PEPTIDES IN HAIR CARE PREPARATION
UTILISATION DE PEPTIDES DE DITHIOL DE THIOREDOXINE, DERIVES OU ANALOGUES DE THIOREDOXINE, DANS DES PREPARATIONS DE SOIN DES CHEVEUX
Patent Applicant/Assignee:
REPLIGEN CORPORATION,
Inventor(s):
PIGIET Vincent P,
Patent and Priority Information (Country, Number, Date):
Patent: WO 8906122 A1 19890713
Application: WO 88US4694 19881229 (PCT/WO US8804694)
Priority Application: US 88353 19880104; US 88354 19880104
Designated States:
(Protection type is "patent" unless otherwise stated - for applications prior to 2004)
AT BE CH DE FR GB IT JP LU NL SE
Publication Language: English
Fulltext Word Count: 6278

Fulltext Availability:
Detailed Description

Detailed Description
... USA 75:5827-5830). Other thioredmin-like peptides include the
class of seed proteins called **purothionins** that have intrinsic
thioredcLdn-like activity (Wada, K and Buchanan, B.B. [1983] in
'Thioredoxins...5 ml aliquots in -200C in O,5M Tris, pH 7.4 with 1 mM
EDTA .

Thioredcodn protein is assayed immunologically using quantitative rocket
immuncelectrophoresis, as described in McEvoy et al...column equilibrated
with OAM Tris, pH 7.5, containing 0.5M NaCl and 1 mM **EDTA** .

The column was washed with two column volumes of the equilibrating buffer
containing 2M urea...

...x 25 cm column of Sephadex"m G 40 equilibrated with 0.05M Tris, 1mM
EDTA , pH 7.4 (TE buffer). The 0.3 ml fractions collected were monitored

at 280...7% (w/w) cammonium bisulfite, 4.65% (w/w) ethanol, and 0.6% (w/w) **polyoxyethylene** (23) lauryl ether. The pH was adjusted to 7.5 with ammonium hydroxide. AU dilutions...30,0
Sodium carbonate glycinate 5eO
Ammonium thioglycollate or
thiolactate (50% aqueous
soln) 3,0
EDTA (disodium salt) 0,3
Sodium p-hydroxybenzoate
methyl ester 0,05
Monoethanolamine 10
Imidazoline. Os2...

15/3,KWIC/64 (Item 1 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

0006236844 BIOSIS NO.: 198886076765
TYROSINE HYDROGEN-BONDING AND ENVIRONMENTAL EFFECTS IN PROTEINS PROBED BY UV RESONANCE RAMAN SPECTROSCOPY
AUTHOR: HILDEBRANDT P G (Reprint); COPELAND R A; SPIRO T G; OTLEWSKI J; LASKOWSKI M JR; PRENDERGAST F G
AUTHOR ADDRESS: DEP CHEM, PRINCETON UNIV, PRINCETON, NEW JERSEY 08544, USA **USA
JOURNAL: Biochemistry 27 (15): p5426-5433 1988
ISSN: 0006-2960
DOCUMENT TYPE: Article
RECORD TYPE: Abstract
LANGUAGE: ENGLISH

...ABSTRACT: chicken [OMCHI3(-)] and from chachalaca [OMCHA(-)], as well as .alpha.1-, .alpha.2-, and .beta.- **purothionin** . At this excitation wavelength interference from phenylalanine is minimized, and it is possible to determine...

...kcal/mol were found for OMCHA3(-) and for .alpha.1- (or .alpha.2-) and .beta.- **purothionin** , respectively. The intensity of the 1176-cm-1 .nu.9a band of Tyr excited at...
...correlate strongly with the estimated H-bond enthalpies, but large deviations are seen for the **purothionins** , reflecting a special environment for the Tyr residue of these proteins, which is believed to ...

...phenylalanine in aqueous solution is about half the value observed in most proteins. Addition of **ethylene** glycol to aqueous phenylalanine increases the intensity, which attains a value similar to those seen...
DESCRIPTORS: CHICKEN CHACHALACA **PUROTHIONIN**

15/3,KWIC/65 (Item 1 from file: 348)
DIALOG(R)File 348:EUROPEAN PATENTS

00642457
HIGH LYSINE DERIVATIVES OF ALPHA-HORDOTHIONIN
DERIVATE VON ALPHA-HORDOTHIONIN MIT HOHEREM BEHALT AN LYSIN
DERIVES D'ALPHA-HORDOTHIONINE A HAUTE TENEUR EN LYSINE
PATENT ASSIGNEE:
PIONEER HI-BRED INTERNATIONAL, INC., (1142078), 800 Capital Square, 400 Locust Street, Des Moines, Iowa 50309, (US), (Proprietor designated states: all)
INVENTOR:
RAO, A. Gururaj, 4628 70th Place, Urbandale, IA 50322, (US)
BEACH, Larry R., 3939 Maquoketa Drive, Des Moines, IA 50311, (US)
LEGAL REPRESENTATIVE:
Irvine, Jonquil Claire et al (74182), J.A. KEMP & CO. 14 South Square

Gray's Inn, London WC1R 5JJ, (GB)
PATENT (CC, No, Kind, Date): EP 745126 A1 961204 (Basic)
EP 745126 B1 010912
WO 9416078 940721
APPLICATION (CC, No, Date): EP 94908585 940112; WO 94US382 940112
PRIORITY (CC, No, Date): US 3885 930113
DESIGNATED STATES: AT; BE; CH; DE; DK; ES; FR; GB; GR; IE; IT; LI; LU; MC; NL; PT; SE
INTERNATIONAL PATENT CLASS: C12N-015/29; C07K-014/00; C12N-005/10; A01H-005/00; A01N-065/00; C12N-001/21
NOTE:
No A-document published by EPO
LANGUAGE (Publication,Procedural,Application): English; English; English
FULLTEXT AVAILABILITY:

Available Text	Language	Update	Word Count
CLAIMS B	(English)	200137	515
CLAIMS B	(German)	200137	492
CLAIMS B	(French)	200137	578
SPEC B	(English)	200137	4391
Total word count - document A			0
Total word count - document B			5976
Total word count - documents A + B			5976

...SPECIFICATION crystal structures have not previously been available for hordothionin or even related compounds such as **purothionin** and viscotoxin. We undertook to develop such structural information.
Three-dimensional modeling of the protein...oil, corn oil and soybean oil; polyols such as propylene glycol, glycerin, sorbitol, mannitol and **polyethylene** glycol; esters such as ethyl oleate and ethyl laurate; agar; buffering agents such as magnesium...

15/3,KWIC/66 (Item 2 from file: 348)
DIALOG(R)File 348:EUROPEAN PATENTS

00579685
BIOCIDAL PROTEINS
BIOZIDE PROTEINE
PROTEINES BIOCIDES
PATENT ASSIGNEE:
Syngenta Limited, (1579446), Fernhurst, Haslemere, Surrey GU27 3JE, (GB), (Proprietor designated states: all)
INVENTOR:
DE BOLLE, Miguel, Kleine Bareelstraat 15 Bus 201, B-2800 Mechelen, (BE)
BROEKAERT, Willem, Frans, Levoldlaan 18, B-1700 Dilbeek, (BE)
CAMMUE, Bruno, Philippe, Angelo, J.B. Woutersstraat 109A, B-1652 Alsemberg, (BE)
VANDERLEYDEN, Jozef, Kastanjelaan 12, B-3001 Heverlee, (BE)
REES, Sarah, Bronwen 32 Micheldever Way, Forest Park Bracknell, Berkshire RG12 3XX; (GB)
LEGAL REPRESENTATIVE:
Roberts, Timothy Wace et al (35342), Intellectual Property Department ZENECA Seeds Jealotts Hill Research Station P.O. Box 3539, Bracknell Berkshire RG12 6YD, (GB)
PATENT (CC, No, Kind, Date): EP 576483 A1 940105 (Basic)
EP 576483 B1 010816
WO 9215691 920917
APPLICATION (CC, No, Date): EP 92906477 920310; WO 92GB423 920310
PRIORITY (CC, No, Date): GB 9105052 910311; GB 9105684 910319
DESIGNATED STATES: AT; BE; CH; DE; DK; ES; FR; GB; GR; IT; LI; LU; MC; NL; SE
INTERNATIONAL PATENT CLASS: C12N-015/82; C07K-014/415; C12N-015/29; A01H-005/00; A01N-065/00; A01N-063/02; A61K-038/00
NOTE:
No A-document published by EPO
LANGUAGE (Publication,Procedural,Application): English; English; English

...poly-D-Lysine HBr, Mastoparan, Defensin NP1, Cathepsin G, Lysozyme, a-hordothionin, b-hordothionin, b- **purothionin** , Stinging nettle lectin, Crotamine, Melittin, Eosinophil major basic protein and Eosinophil cationic protein.
Preferred proteins...
...D-Lysine HBr, Mastoparan, Kassinin, Defensin NP1, Cathepsin G, Lysozyme, a-hordothionin, b-hordothionin, b- **purothionin** , Stinging nettle lectin, Crotamine, Melittin and Eosinophil cationic protein.
Preferred proteins for killing or inhibiting...

...hormone 1-24, Citrate synthase, Defensin NP1, Cathepsin G, Lysozyme, a-hordothionin, b-hordothionin, b- **purothionin** , Crotamine, Melittin, Eosinophil major basic protein, Eosinophil cationic protein, and Eosinophil peroxidase.
Example 2 - Protein...proteins and enzymes Citrate Synthase, Defensin 1, Cathepsin G, Lysozyme, a-Hordothionin, b-Hordothionin, b- **Purothionin** , Stinging Nettle Lectin, Crotamine, Melittin, Eosinophil Major Basic Protein, Eosinophil cationic Protein and Eosinophil Peroxidase...

...CLAIMS selected from: Defensin NP1, Magainin-2, Magainin-A, Magainin-G, a-Hordothionin, b-Hordothionin, b- **Purothionin** , poly-L-Lysine HBr, poly-L-Lysine HCl, poly-D-Lysine, poly-D-Lysine HBr...

15/3,KWIC/68 (Item 4 from file: 348)
DIALOG(R)File 348:EUROPEAN PATENTS

00245328
Therapeutic and related uses of dithiol peptides.
Therapeutische und verwandte Verwendungen von Dithiol-Peptiden.
Utilisations therapeutiques et apparentees des dithiol peptides.
PATENT ASSIGNEE:
REPLIGEN CORPORATION, (545550), 101 Binney Street, Cambridge Massachusetts 02142, (US), (applicant designated states: BE;CH;DE;ES;FR;GB;GR;IT;LI;NL;SE)
INVENTOR:
Pigiet, Vincent P., 666 Main Street, Apt. L-1, Winchester Massachusetts 01898, (US)
Millis, Cynthia D., 3720 West 8th Avenue, Apt. 5, Vancouver British Columbia, V6R 1Z3, (CA)
LEGAL REPRESENTATIVE:
Perry, Robert Edward et al , GILL JENNINGS & EVERY 53-64 Chancery Lane, London WC2A 1HN, (GB)
PATENT (CC, No, Kind, Date): EP 237189 A2 870916 (Basic)
APPLICATION (CC, No, Date): EP 87301150 870210;
PRIORITY (CC, No, Date): US 839857 860314; US 921287 861020; US 828112 860210
DESIGNATED STATES: BE; CH; DE; ES; FR; GB; GR; IT; LI; NL; SE
INTERNATIONAL PATENT CLASS: A61K-037/02;
ABSTRACT WORD COUNT: 18

LANGUAGE (Publication,Procedural,Application): English; English; English
FULLTEXT AVAILABILITY:

Available Text	Language	Update	Word Count
CLAIMS A	(English)	EPABF1	285
SPEC A	(English)	EPABF1	3897
Total word count - document A			4182
Total word count - document B			0
Total word count - documents A + B			4182

...SPECIFICATION i.e., rates of reaction of uncatalyzed reactions are slow). Certain iron-chelators such as **ethylenediaminetetraacetic** acid (**EDTA**) promote the iron-catalyzed Haber-Weiss reaction presumably by chelating the Fe(sup 3)(sup...or one that is readily soluble can be utilized. For example, cocoa butter and various **polyethylene** glycols (Carbowaxes) can serve as the vehicle.

For intranasal instillation, fluid unit dosage forms are...USA, 75, 5827-5830). Other thierodoxin-like peptides include the class of seed proteins called **purothionins** that have intrinsic thioredoxin-like activity (Wada, K. and Buchanan, B.B. (1983) in "Thioredoxins...show that thioredoxin chelates iron with an affinity comparable to or better than that of **EDTA** . Similar results were also observed with copper.
Example 4
Upon substituting the thioredoxin in Examples...

...essentially the same results.
Example 6
Thioredoxin was compared to several iron chelators (i.e., **EDTA** and desferrioxamine) in its ability to prevent radical formation. The iron caralyzed formation of the...

...M inhibited all catalytic activity of the iron in that no formaldehyde was detected, whereas **EDTA** at 250 (mu)M gave a rate of 7.1 (+-) 0.8 nmols formaldehyde/30...

...nmols of formaldehyde/30 min. Thus thioredoxin behaves as desferrioxamine in inhibiting radical formation whereas **EDTA** cannot.
Example 7
Thioredoxin inhibited the peroxidation of arachidonic acid micelles in an iron-catalyzed...

15/3,KWIC/69 (Item 5 from file: 348)
DIALOG(R)File 348:EUROPEAN PATENTS

00224186
Protein-folding enzyme.
Protein faltendes Enzym.
Enzyme de pliage de proteines.
PATENT ASSIGNEE:
REPLIGEN CORPORATION, (545550), 101 Binney Street, Cambridge Massachusetts 02142, (US), (applicant designated states: BE;CH;DE;FR;GB;IT;LI;NL;SE)
INVENTOR:
Pigiet, Vincent P., 666 Main Street Apt. L-1, Winchester Massachusetts 01898, (US)
Rusche, James R., 21 Varnum Street, Worcester Massachusetts 01603, (US)
Schuster, Barbara J., 3520 Plaza Drive, State College Pennsylvania 16801, (US)
LEGAL REPRESENTATIVE:
Perry, Robert Edward et al , GILL JENNINGS & EVERY 53-64 Chancery Lane, London WC2A 1HN, (GB)
PATENT (CC, No, Kind, Date): EP 225156 A2 870610 (Basic)
EP 225156 A3 890111
APPLICATION (CC, No, Date): EP 86309188 861125;
PRIORITY (CC, No, Date): US 802569 851127; US 894421 860808
DESIGNATED STATES: BE; CH; DE; FR; GB; IT; LI; NL; SE
INTERNATIONAL PATENT CLASS: C12N-009/00; C12N-015/00; C12P-021/02; C07K-003/08;
ABSTRACT WORD COUNT: 70

LANGUAGE (Publication,Procedural,Application): English; English; English
FULLTEXT AVAILABILITY:

Available Text	Language	Update	Word Count
CLAIMS A	(English)	EPABF1	452
SPEC A	(English)	EPABF1	5235
Total word count - document A			5687
Total word count - document B			0
Total word count - documents A + B			5687

...SPECIFICATION USA, 75:5827-5830). Other thioredoxin-like peptides include the class of seed proteins called **purothionins** that have

intrinsic thioredoxin-like activity (Wada, K. and Buchanan, B.B. (1983) in "Thioredoxins...YM10 filter (Amicon, Danvers, MA). The buffer was exchanged with 50 mM Tris, 3 mM **EDTA** , pH 7.4 by diluting and concentrating the sample. The sample was stored at 4...enzyme in 0.1 M Tris, pH 7.4 or 9.0 with 1 mM **EDTA** containing various amounts of thioredoxin shufflease or thioredoxin. At various times aliquots were assayed and...

...diluting the inactive RNase into 0.1 M Tris, pH 7.4 with 1 mM **EDTA** containing various amounts of thioredoxin shufflease and/or reduced DTT. At various times aliquots were...6X SSC (1X SSC = 0.15 M NaCl, 0.015 M sodium citrate, 1 mM **EDTA**) and 10X Denhardt's solution (100 X - 2% bovine serum albumin, 2% ficoll, 2% polyvinyl...Reduced and Denatured RNase

At pH 9.0 (0.1 M Tris, 1.0 mM **EDTA**) thioredoxin shufflease or a mixture of thioredoxin shufflease and oxidized DTT increased the rate of ...

...denatured ribonuclease than is thioredoxin.

At pH 7.4 (0.1 M Tris, 1 mM **EDTA**) thioredoxin shufflease significantly increased the rate of refolding as compared to air oxidation. The time...

...said protein.

Example 18

At pH 8.5 (0.1 M Tris, 1.0 mM **EDTA**) thioredoxin shufflease increased the rate of reactivation of scrambled RNase as compared to air oxidation ...

15/3,KWIC/70 (Item 6 from file: 348)

00220520

Folding disulfide-cross-linkable proteins.
In Falten gelegte Proteine, durch Disulfid gebunden.
Proteines pliantes liees par pont disulfure en croix.

PATENT ASSIGNEE:
REPLIGEN CORPORATION, (545550), 101 Binney Street, Cambridge Massachusetts 02142, (US), (applicant designated states: BE;CH;DE;FR;GB;IT;LI;NL;SE)
INVENTOR:
Pigiet, Vincent P., 666 Main Street Apt. L-1, Winchester Massachusetts 01898, (US)
Schuster, Barbara J., 3520 Plaza Drive, State College Pennsylvania 16801, (US)
LEGAL REPRESENTATIVE:
Perry, Robert Edward et al , GILL JENNINGS & EVERY 53-64 Chancery Lane, London WC2A 1HN, (GB)

PATENT (CC, No, Kind, Date): EP 208539 A2 870114 (Basic)
EP 208539 A3 880803
APPLICATION (CC, No, Date): EP 86305272 860708;
PRIORITY (CC, No, Date): US 753848 850711; US 812162 851223; US 859595 860505
DESIGNATED STATES: BE; CH; DE; FR; GB; IT; LI; NL; SE
INTERNATIONAL PATENT CLASS: C07K-015/00; C07K-015/12; C12P-021/00; C07K-007/00;
ABSTRACT WORD COUNT: 59

LANGUAGE (Publication,Procedural,Application): English; English; English
FULLTEXT AVAILABILITY:

Available Text	Language	Update	Word Count
CLAIMS A	(English)	EPABF1	515
SPEC A	(English)	EPABF1	4071
Total word count - document A			4586
Total word count - document B			0

Total word count - documents A + B 4586

...SPECIFICATION USA, 75, 5827-5830). Other thioredoxin-like peptides include the class of seed proteins called **purothionins** that have intrinsic thioredoxin-like activity (Wada, K. and Buchanan, B.B. (1983) in "Thioredoxins...by diluting the reduced enzyme in 0.1M Tris, pH 7.4 with 1 mM **EDTA** containing various amounts of thioredoxin and/or oxidized DTT. At various times aliquots were assayed...

...diluting the reduced enzyme in 0.1 M Tris, pH 7.5 with 1 mM **EDTA** to a final concentration of 340 (mu)g/ml. Oxidized thioredoxin (100 (mu)M: was ...pH 7.5 or 0.05 M Tris, pH 9.0, each containing 1 mM **EDTA** . The regenerating system consisted of a catalytic amount of thioredoxin reductase and an excess (1...diluting the inactive RNase into 0.1 M Tris, pH 7.4 with 1 mM **EDTA** containing various amounts of oxidized thioredoxin preincubated with reduced DTT 30 min prior to addition...

?logoff hold

26feb05 10:16:39 User228206 Session D2370.5

$0.02 0.006 DialUnits File155
$0.21 1 Type(s) in Format 9
$0.21 1 Types
$0.23 Estimated cost File155
$3.71 0.629 DialUnits File654
$21.00 30 Type(s) in Format 3
$21.00 30 Types
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$0.04 Estimated cost File399
$2.39 0.504 DialUnits File349
$51.20 32 Type(s) in Format 3
$51.20 32 Types
$53.59 Estimated cost File349
$0.05 0.003 DialUnits File340
$0.05 Estimated cost File340
$0.03 0.006 DialUnits File5
$2.00 1 Type(s) in Format 3
$2.00 1 Types
$2.03 Estimated cost File5
$0.22 0.049 DialUnits File348
$10.20 6 Type(s) in Format 3
$10.20 6 Types
$10.42 Estimated cost File348
$0.03 0.003 DialUnits File347
$0.03 Estimated cost File347
$0.01 0.003 DialUnits File65
$0.01 Estimated cost File65
$0.01 0.003 DialUnits File35
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$0.06 Estimated cost File357
$0.03 0.003 DialUnits File73
$0.03 Estimated cost File73
$0.01 0.003 DialUnits File50
$0.01 Estimated cost File50
OneSearch, 18 files, 1.230 DialUnits FileOS
$0.26 TELNET
$91.66 Estimated cost this search